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ENDOCRINOLOGY

VOLUME 44 JANUARY, 1949 NUMBER 1

EFFECT OF ANDROGEN AND ESTROGEN ON SUCCINIC DEHYDROGENASE AND CYTOCHROME OXIDASE OF RAT PROSTATE AND SEMINAL VESICLE[1]

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PREVIOUS work in this laboratory indicates that in ovarian tissues correlations can be made between the functional state of the tissues and fluctuations in activity of such enzymes as succinic dehydrogenase (SDH-ase) (Meyer, Soukup, McShan, Biddulph, 1947), malic dehydrogenase and cytochrome oxidase (McShan, Erway, Meyer, 1948), adenosine triphosphatase (Biddulph, Meyer, McShan, 1946), and acid and alkaline phosphatase (Stafford, McShan, Meyer, 1947). The experiments to be reported here were undertaken to determine whether such a relationship exists in the prostate and seminal vesicle with respect to SDH-ase and cytochrome oxidase following castration and replacement therapy.

MATERIALS AND METHODS

The animals used were Sprague-Dawley male rats ranging from 200 to 300 gm. body weight. Of a total of 55 animals, 7 were set aside as normal controls and did not receive any treatment. One animal was used for each experiment; 5 for SDH-ase controls and 2 for cytochrome oxidase. The remainder were castrated and divided into 3 groups.

Group I consisted of 20 animals which did not receive any injections and were sacrificed at intervals of 1, 4, and 6 days after castration. In the 1-day category, sufficient tissue was obtained from one animal for one experiment, but on the 4th and 6th days after castration atrophy of the prostate was great enough to require combining the glands from 2 animals.

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Group II contained 14 animals which received 2 subcutaneous injections per day, 12 hours apart. Each injection consisted of 300 μg. testosterone propionate dissolved in 0.2 cc. of corn oil. The first injection was given at the time of castration and the animals were sacrificed at intervals of 1, 4, and 6 days after castration.

Group III consisted of 14 animals which received the same dosage of testosterone propionate as group II plus simultaneous injections of 100 μg. of estradiol dipropionate dissolved in 0.2 cc. of corn oil. As in group II, the first injections were given at the time of castration and the animals were sacrificed at 1, 4, and 6 days after castration.

After killing the animals by cervical dislocation the ventral lobe of the prostate and both seminal vesicles were dissected out. The tissues were thoroughly chopped with a sharp scalpel blade, rinsed in ice-cold glass-distilled water, blotted dry on analytical grade filter paper and weighed. This procedure was followed in order to remove as much of the glandular secretions as possible. Pieces of tissue weighing 50 to 200 mg. were homogenized and diluted with glass-distilled water to make either 5% or 1% homogenates, the material being kept ice-cold throughout.

The procedures described by Potter and Schneider (1942) and Schneider and Potter (1943) for SDH-ase and cytochrome oxidase were used in these experiments. The 5% homogenates were used for SDH-ase determinations, but due to the high activity of the tissues 1% homogenates had to be used for cytochrome oxidase. The results of each determination were calculated using the dry weight of the homogenate used for that determination. Results are given in terms of Q_{O_2}, or mm^3 of oxygen consumed/mg. of dry tissue/hour. Duplicate flasks were used in making all determinations in the Warburg apparatus.

RESULTS AND DISCUSSION

Examination of Figs. 1 and 2 shows that, following castration, the decline in SDH-ase activity roughly parallels the loss in gland weight. The weight loss in the prostate is fairly uniform between 0 and 6 days while the enzyme activity curve shows no change on day 1 and a sharp drop between days 1 and 4. In the seminal vesicle the weight loss is fairly uniform between days 0 and 6 and the decline in enzyme activity is almost linear between days 0 and 4. Thus the SDH-ase appears to react to an absence of androgen more quickly in the seminal vesicle than in the prostate.

A comparison of these results with the observations of Moore, Price, and Gallagher (1936) and Moore, Hughes, and Gallagher (1936) on the morphological changes in the prostate and seminal vesicle following castration, provides an interesting correlation. These authors found that within 24 hours after castration the height of the secretory cells of the seminal vesicle was reduced to nearly one half of the normal value and almost no secretory granules remained in the cytoplasm. Similar changes occurred in the secretory cells of the prostate but not until the fourth day following castration. Simultaneously with these cytological changes there was a decrease in the size of the lumina of the prostatic tubules and definite changes in the appearance of the

secretion in the seminal vesicle. Changes in the mitochondria occurred but were indefinite. Our data show that there occurs a decline in enzyme activity at about the same time as the loss of secretory granules from the epithelial cells of these tissues and a weight decrease which,

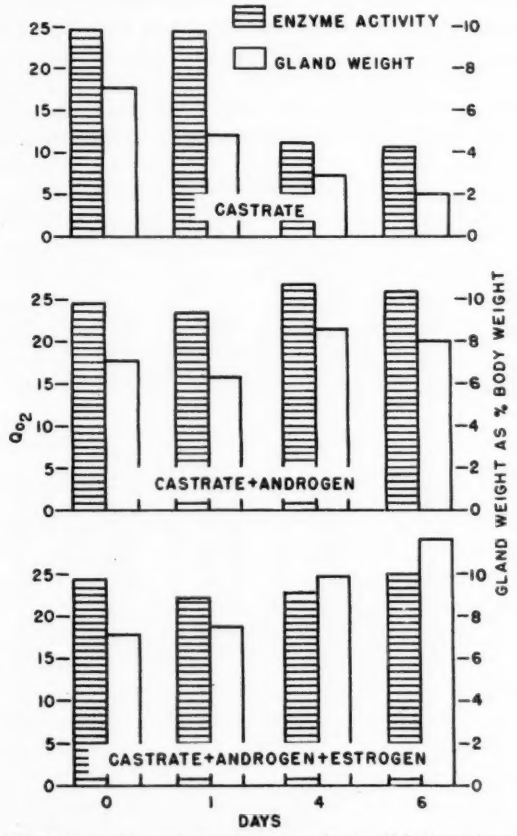

FIG. 1. Effect of testosterone propionate and estradiol dipropionate on the weight and succinic dehydrogenase activity of the prostate.

at least in the case of the seminal vesicle, follows closely the involution of the secretory epithelium.

The ability of the synthetic androgens to maintain the functional and morphological integrity of the male sex accessories in the absence of the testes is well known. Our data show that testosterone propionate, in an approximately physiological dose, will likewise maintain the SDH-ase activity of the accessories at a normal level. The dosage used did not prevent a 23% loss in weight of the prostate during the first day but did prevent such loss in the seminal vesicle.

While the enzyme activity showed a slight increase over normal in both tissues, the increase was somewhat greater and more constant in the seminal vesicle. The observations of Moore *et al.* discussed above and those of Korenchevsky, Dennison and Brovsin (1936) indicate that on a morphological basis the seminal vesicle is more sensitive to the presence or absence of androgen than the prostate. The data presented here show that the same is true with respect to SDHase activity.

The simultaneous administration of estrogen and androgen produced striking effects on the gland weight. The prostate showed a

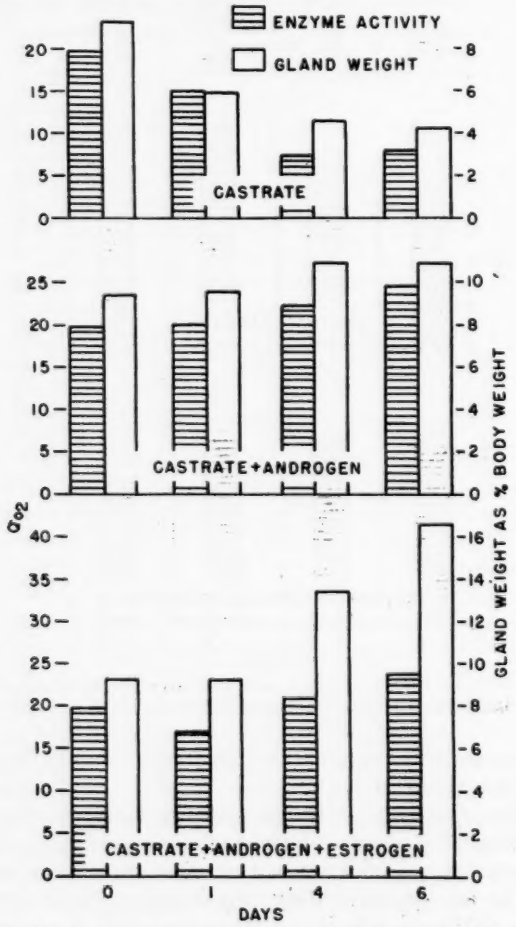

FIG. 2. Effect of testosterone propionate and estradiol dipropionate on the weight and succinic dehydrogenase activity of the seminal vesicle.

slight depression on day 1, not as great, however, as with androgen alone, followed by an almost linear increase to 41% above normal on day 6. The weight of the seminal vesicle underwent no change on day 1 but increased linearly to 77% above normal on day 6.

There is excellent evidence that estrogens stimulate growth of the fibromuscular layer in the male accessories and to a slight extent that of the secretory epithelium, especially in the seminal vesicle (Moore, Hughes and Gallagher, 1936; Korenchevsky, Dennison and Brovsin, 1936; Korenchevsky and Dennison, 1935; Overholser and Nelson, 1935; Tislowitz, 1939). This appears to be a logical explanation for the greater part of the weight increase which we found in this group of animals. However, consideration of the weight changes for tissues treated with androgen alone indicates that the androgen was responsible for some of this increase.

The combined effect of estrogen and androgen on enzyme activity was quite different from that exerted on gland weight. In the prostate, activity was depressed 10% below normal until day 6 when it returned to the normal level. In the seminal vesicle there was a 14% decrease in activity on day 1 followed by a linear rise to 21% above normal on day 6.

Comparison of these results with those obtained using androgen alone reveals that, in the seminal vesicle, the activity is no greater on day 6 than that obtained with androgen alone. On day 1, however, there was a definite depression which was not obtained with androgen alone. In the prostate the activity remained below that obtained with androgen alone throughout the experimental period. However, reference to Table 1 will show that the ranges of values obtained for these two groups overlap in several places. Hence the significance of these differences is open to question and one hesitates to conclude that androgens and estrogens are antagonistic in their effect on male accessories. The data on gland weights in these two groups are open to the same criticism except for the 6-day seminal vesicles; here the results indicate a definite synergism between the two hormones.

The cytochrome oxidase data, though not extensive, suggest that the activity of this enzyme follows much the same general pattern as SDH-ase. This might be expected since cytochrome oxidase is a part of the SDH-ase system and previous work in this laboratory has shown this to be true in other endocrine tissues.

SUMMARY

The effects on the seminal vesicle and prostate of castration, castration followed by androgen injections, and castration followed by androgen and estrogen injections have been studied in the rat. Changes in activity of succinic dehydrogenase, cytochrome oxidase, and gland weights were determined for each of these conditions and compared

TABLE I. SUCCINIC DEHYDROGENASE AND CYTOCHROME OXIDASE ACTIVITY OF RAT PROSTATE AND SEMINAL VESICLE

		Succinic dehydrogenase						Cytochrome oxidase			
		1 Day		4 Days		6 Days		4 Days		6 Days	
Treatment		Prostate	Sem. Ves.	Prostate	Sem. Ves.	Prostate	Sem. Ves.	Prostate	Sem. Ves.	Prostate	Sem. Ves.
None	Q_{O_2}	24.4 (23.1–27.5)	19.8 (16.5–22.8)					274 (257–291)	147 (142–152)		
	Weight[2] %	7.16 (6.32–8.44)	9.3 (6.7–12.0)					6.9 (6.3–7.4)	9.4 (9.2–9.6)		
	Weight mg.	200 (158–249)	235 (197–285)					172 (158–186)	235 (229–241)		
	No. Expts.	5	5					2	2		
Castrate	Q_{O_2}	24.1 (18.2–29.3)	15.0 (11.9–18.2)	11.1 (9.4–13.3)	7.3 (4.6–10.2)	10.9 (8.5–14.1)	7.9 (5.6–11.9)	190	95	190	75
	Weight %	4.8 (3.8–6.4)	5.9 (4.4–7.6)	2.9 (2.4–3.1)	4.6 (3.6–5.5)	2.0 (1.2–3.1)	4.3 (3.6–5.7)	3.1	5.2	1.9	3.8
	Weight mg.	133 (89–191)	163 (104–226)	94 (58–78)	102 (55–155)	58 (34–89)	106 (50–167)	88	147	50	103
	No. Expts.	4	4	4	4	3	3	1	1	1	1
Castrate plus androgen	Q_{O_2}	23.5 (20.5–25.6)	20.0 (17.6–22.0)	26.9 (25.2–27.5)	22.1 (17.2–26.0)	26.0 (21.4–29.8)	24.4 (21.8–27.4)	284	109	291	112
	Weight %	6.3 (3.6–8.3)	9.5 (7.4–10.6)	8.6 (5.8–13.3)	10.8 (7.3–15.7)	8.0 (5.2–12.5)	10.8 (8.8–11.9)	5.8	7.2	5.2	11.1
	Weight mg.	150 (96–190)	224 (200–243)	298 (210–360)	377 (277–432)	206 (150–237)	301 (270–338)	200	253	182	390
	No. Expts.	4	4	4	4	5	5	1	1	1	1
Castrate plus androgen plus estrogen	Q_{O_2}	22.0 (20.0–25.5)	17.0 (15.9–19.2)	22.6 (19.6–24.5)	20.9 (18.1–22.0)	24.9 (21.9–28.8)	23.9 (20.5–27.5)	288	162	331	210
	Weight %	7.4 (6.9–8.0)	9.3 (6.8–12.3)	9.8 (7.4–13.8)	13.3 (12.2–15.2)	11.6 (9.5–12.7)	16.6 (13.9–18.7)	13.7	12.1	12.7	13.9
	Weight mg.	189 (183–200)	234 (179–295)	220 (177–281)	328 (304–365)	250 (228–292)	391 (367–412)	440	389	394	432
	No. Expts.	4	4	5	5	4	4	1	1	1	1

[2] Values in this line were calculated as $\frac{\text{gland weight}}{\text{body weight}} \times 10^{-4}$.

to the normal. Castration caused a significant decrease in both enzyme activity and gland weight while the administration of androgen maintained these factors at the normal level. The simultaneous administration of androgen and estrogen caused a significant rise above normal of both factors in the seminal vesicle but only of the gland weight in the prostate. The possible relationship of these findings to the morphology and function of the glands is discussed.

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OBSERVATIONS ON THE EFFECTS OF PREPARTAL AND POSTPARTAL ESTROGEN AND PROGESTERONE TREATMENT ON LACTATION IN THE RAT

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NUMEROUS attempts to determine the relation of hormones to the initiation of lactation following parturition have been made. The onset of lactation has been attributed to removal of an inhibitory action of progesterone, estrogen or a combination of progesterone and estrogen. The establishment of lactation following the removal of corpora lutea in the rabbit (Hammond, 1917) and following the removal of luteinized ovaries in the rat (Selye, Collip and Thomson, 1933) suggested an inhibitory action of the secretion of the corpora lutea but injection of extract of the corpora lutea (Anselmino and Hoffmann, 1936) and progesterone (Folley and Kon, 1937a) in lactating rats failed to inhibit established lactation. The report of Parkes and Bellerby (1927) demonstrated an inhibitory action of estrogens on established lactation in the rat with intact ovaries. Folley and Kon (1937b), Edelmann and Gaunt (1941) and Barsantini and Masson (1947) have shown that inhibition of established lactation by estrogens is more effective in normal than in ovariectomized rats. Fauvet (1941) found that simultaneous injection of estrogen and progesterone produced marked inhibition of established lactation in ovariectomized rats while estrogen alone induced no inhibition.

Two studies have been made on the effect of prepartal injection of estrone on lactation. Smith and Smith (1933) found that the initiation of lactation was prevented in the rabbit by estrone administration begun on the 20th day of pregnancy. Edelmann and Gaunt (1941) reported that estrone treatment started 4 days before delivery in the normal rat did not prevent initiation of lactation although it brought about the inhibition of established lactation a few days earlier than did similar treatment started on the day of delivery. We are not aware of any observations on the effect of prepartal administration of progesterone or of a combination of progesterone and estrogen on the initiation of lactation. Therefore, the present study was planned to observe the effects of prepartal injection of estrogen alone, progesterone alone

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and various combination of these hormones on the initiation of lactation in the rat and to compare these results with the effects obtained by similar hormone treatment after the establishment of lactation.

METHODS

Daily subcutaneous injections were given to the mothers and the growth rate of the suckling young was used as the index of the rate of lactation (cf. Cowie and Folley, 1947). Uniparous rats were used in all experiments and six rats per litter were provided at the beginning of the lactation period, where the lactation period is defined as beginning at delivery. In the ovariectomized cases the operation was carried out on the day of parturition or the first day thereafter under ether anaesthesia. In those animals showing prolonged pregnancy as a result of prepartal hormone treatment the young were removed on the 22nd day of pregnancy through a mid-line incision by hysterectomy under ether or local procaine anaesthesia. Days of pregnancy were counted from the day that sperms were found in the vaginal smear. The duration of pregnancy was normally 22 days in the strain of rats used in this study.

RESULTS

The growth rates of 85 litters containing 6 young rats per litter were observed in this study. The mothers of 63 of these litters were given hormone injections; the mothers of 22 litters were untreated controls. The average weights of the young are shown in Table 1. The living litters included in the table are those with all 6 members surviving, it being expected that the death of even 1 member would alter the supply of milk for the remaining members.

Effects of estrogen. Daily injection of 1 mg. of diethylstilbestrol dipropionate begun on the second day of lactation brought about a marked decrease of lactation in 4 normal and in 4 ovariectomized rats while treatment with 200γ of this substance per day inhibited lactation in 4 normal animals although it did not produce inhibition in 3 out of 4 ovariectomized rats (Table 1, section 1). All mothers treated with diethylstilbestrol dipropionate and those given 200γ of estrone lost weight during the lactation period. All other treated mothers and the untreated controls gained weight.

The results in section 2 of Table 1 show that established lactation was not inhibited in 3 ovariectomized rats given 200γ or in 4 ovariectomized rats given 100γ of estrone daily from the second to the 16th day of lactation, while a variable extent of inhibition was obtained in normal animals during daily injection of either 100γ, 25γ or 5γ of estrone. The onset of inhibition occurred 10 to 12 days after the beginning of estrone administration.

Estrone treatment begun before parturition did not prevent the initiation of lactation though depression of established lactation did occur about the 10th to 12th day after parturition in all mothers showing well formed corpora lutea (Table 1, section 3). In one animal injection of 100γ of estrone daily was begun on the 20th day of preg-

nancy. Although the young were delivered 1 day premature they were all viable and lactation was promptly established. The young were growing at a normal rate up to the 6th day of lactation when they were killed by the mother. In an effort to avoid premature delivery the daily dosage of estrone was reduced to 25γ and injection was begun on the 19th day of pregnancy in 3 rats. The first of these failed to give birth to the young and died in labor. Twelve hours after the second animal began to show bleeding and labor movements the young were removed by hysterectomy. Injection of estrone was continued and on the 6th day after the operation normal lactation was established and continued normally up through the 14th day but thereafter lactation was markedly depressed. In the third animal the young were delivered by hysterectomy 24 hours after the first 25γ injection of estrone; they were premature and did not survive. This animal accepted the young from a foster mother and lactation was promptly established. It should be pointed out that the ovaries were removed from this last animal at the time of hysterectomy and that lactation continued normally throughout the period of estrone treatment. Daily injection of 25γ of estrone was started on the 21st day of pregnancy in 4 rats. Although initiation of lactation was not prevented, marked depression of lactation occurred in 3 of these 4 rats about the 10th day after delivery. The other animal showed no depression of lactation throughout the experiment. It is interesting to note that large ovaries with well-developed corpora lutea (fig. 5) were found in the 3 animals showing depression of lactation and that small ovaries with atrophic corpora lutea (fig. 6) were found in the rat showing no depression of lactation.

Effects of progesterone. Neither 2 mg. nor 5 mg. doses of progesterone per day inhibited lactation in intact rats when treatment was started on the second day of lactation (Table 1, section 4). To arrive at a dosage of progesterone adequate for study of the effects on initiation of lactation the amount of this hormone required to prevent the onset of parturition was determined. It was found that parturition never occurred, although some uterine bleeding was seen, in 3 rats given 1 mg. of progesterone daily beginning on the 19th day of pregnancy. Labor was observed in 1 of these animals. When the dosage was increased to 2.5 mg. no uterine bleeding appeared and the animals showed no signs of labor. The young were delivered by hysterectomy on the 22nd day of pregnancy and daily injections were continued for 10 days after the operation. The lactation rate was approximately the same as that of control animals similarly operated but not treated with progesterone (cf. sections 5 and 10 of Table 1). The slow rate of growth observed in the young of both groups of operated animals during the first 5 postoperative days is apparently due to operative trauma.

Effects of simultaneous treatment with estrone and progesterone. In 3

TABLE 1. MEAN VALUES ON THE GROWTH RATES OF YOUNG IN LITTERS CONTAINING 6 MEMBERS DURING VARIOUS TREATMENTS OF THE MOTHER

Daily treatment of mother				Condition of mother	No. of litters	Initial wt.	5th day		10th day		13th day		16th day	
Substance and section		Dose	Period				wt.	litters living†	wt.	litters living†	wt.	litters living†	wt.	litters living†
						gm.	gm.	%	gm.	%	gm.	%	gm.	%
S	1	1 mg.	2L–16L	N	4	6.1	9.3	100	11.7	75	13.6	50	15.9	25
		1 mg.	2L–16L	O	4	5.7	9.0	100	12.3	100	14.5	75	17.4	50
		200γ	2L–16L	N	4	6.3	9.3	100	12.4	75	11.6	75	15.7	25
		200γ	2L–16L	O	4	6.3	10.3	100	16.1	75	19.1	75	21.3	75
E	2	200γ	2L–16L	O	3	6.0	9.8	100	14.1	100	17.5	100	21.2	100
		100γ	2L–16L	N	2	6.6	10.3	100	13.2	100	17.6	50	18.8	50
		100γ	2L–16L	O	4	6.9	9.9	100	16.2	100	20.0	100	22.8	100
		25γ	2L–16L	N	3	6.7	12.2	100	18.5	100	18.7	67	18.0	67
		5γ	2L–16L	N	2	6.0	9.3	100	12.3	100	13.0	100	13.1	100
E	3	100γ	20P– 5L	S.D.	1	5.1	8.9	100						
		25γ	19P–16L	H	1	6.6	7.4	100	13.9	100	18.3	100	18.0	100
		25γ	19P–16L	H and O	1	7.3	12.0	100	18.7	100	22.7	100	26.6	100
		25γ	21P–14L	S.D.	3	6.4	13.2	100	18.2	100	18.7	67		
		25γ	21P–16L	S.D.*	1	6.6	11.5	100	16.9	100	19.5	100	24.2	100
P	4	2 mg.	2L–16L	N	3	6.5	10.9	100	17.1	100	20.5	100	24.6	100
		5 mg.	2L–16L	N	2	6.3	10.2	100	16.7	100	19.4	100	21.8	100
P	5	2.5 mg.	19P–10L	H	3	6.1	6.9	100	12.0	100	15.6	100	19.3	100
E P	6	100γ 2.5 mg.	2L–16L	O	3	6.4	10.9	100	16.0	100	18.2	100	18.3	100
E P		25γ 2.5 mg.	2L–16L	O	4	6.5	11.5	100	17.8	100	20.2	100	20.4	100
E P		10γ 2.5 mg.	2L–16L	O	7	6.5	9.8	100	15.3	100	17.5	100	17.9	100
E P	7	100γ 1 mg.	20P–16L	H and O	2	6.7	9.1	100	14.7	100	17.6	100	20.0	100
E P		25γ 2.5 mg.	20P–16L	H and O	2	6.1	7.7	100	11.4	100	13.2	100	14.3	100
none	8			N	13	6.4	10.6	100	17.4	100	21.1	100	25.2	100
none	9			O	7	6.1	9.5	100	15.8	100	19.6	100	23.8	100
none	10			H	2	6.0	7.1	100	12.2	100	16.2	100	20.1	100

S = diethylstilbestrol dipropionate.
E = estrone.
P = progesterone.
2L = second day of lactation.
20P = twentieth day of pregnancy.
N = intact ovaries.
O = ovariectomized.
S.D. = spontaneous delivery.
H = removal of young by hysterectomy.

* This animal showed atrophic ovaries at autopsy (see fig. 6).
† Litters with all members surviving.

ovariectomized rats daily amounts of progesterone (2.5 mg.) and estrone (100γ), without effect when given separately, were injected simultaneously, beginning on the 2nd day of lactation. The data in section 6 of Table 1 show that lactation was markedly depressed in these 3 animals after the 13th day. Similar results were obtained when the same dosage of progesterone (2.5 mg. daily) was given in combination with 25γ or with 10γ of estrone.

Two combinations of estrone and progesterone were used for simultaneous injections beginning on the 20th day of pregnancy (Table 1, section 7). Viable young were delivered by hysterectomy 48 hours after the first injection and ovariectomy was done at the same time. In 2 rats injected with 100γ of estrone and 1 mg. of progesterone lactation was established promptly and it was equal to that of the operated controls throughout the period of observation. In 1 animal given 25γ of estrone and 2.5 mg. of progesterone the initiation of lactation was retarded but a slightly subnormal rate was established by the 7th day after the operation, with further inhibition occurring on the 12th day. In another animal receiving 25γ of estrone and 2.5 mg. of progesterone an incrustation of the nipples developed which obstructed their openings. This horny covering was sloughed off on the 6th day after hysterectomy, but lactation was not established until the 13th day although the animal had nursed young from foster mothers almost continuously from the time of the operation. Lactation continued at a normal rate until the 26th day after hysterectomy (13th day of lactation) when marked inhibition occurred. The 13th day after hysterectomy was taken arbitrarily as the first day of lactation for this animal in order to show in the table the effects of continued hormone treatment on established lactation.

Histological changes in the mammary gland. Sections of the mammary gland in animals of the various treated groups were made on the 17th to 19th day of lactation. Findings typical of the various groups are shown in figures 1 to 4. The alveolar sacs were large and well filled with milk in the ovariectomized rats given 100γ of estrone and the alveolar cells appeared normal (fig. 1). On the other hand, the alveolar sacs were small and frequently almost devoid of milk in the intact rats given 100γ of estrone and the alveolar cells usually showed proliferation (fig. 2). In the ovariectomized rats given estrone and progesterone simultaneously the alveoli were usually small and the alveolar cells frequently formed walls several layers thick indicating pronounced proliferation (figs. 3 and 4). It should be noted that 25γ of estrone (fig. 4) was as effective as 100γ (fig. 3) in bringing about proliferation when these amounts were given with 2.5 mg. of progesterone.

DISCUSSION

The results indicate that neither estrogens alone nor progesterone alone inhibits established lactation or prevents the initiation of lacta-

tion. A large number of reports indicates that estrogens inhibit lactation. It is suggested that the findings in many of these studies may be reconciled with our observations that estrone neither prevents nor inhibits lactation in the ovariectomized rat if the following assumptions are made: (1) Inhibition of lactation in estrogen-treated intact animals is due to a synergistic action of the injected estrogen with an ovarian secretion, probably progesterone. (2) Inhibition of lactation in ovariectomized animals requires dosages of estrogen which greatly exceed the normally occurring secretion. (3) the stimulation resulting from nursing or manipulation of the mammary gland and from complete removal of the milk is essential to maximal lactation.

The assumption that inhibition of lactation by estrogen treatment in intact animals is due to a synergism with another ovarian secretion is supported by the observation in this report that simultaneous injection of progesterone and estrone inhibits lactation in ovariectomized rats. Since 5γ of estrone per day markedly depresses lactation in intact rats while a 200γ dosage shows no inhibitory effect in ovariectomized animals it is clear that the inhibition observed in intact animals is not due to estrogens alone, and it is demonstrated that a synergistic action with some other ovarian hormone is occurring. The failure of 25γ of estrone to produce any depression of lactation in a rat showing almost complete absence of corpora lutea (fig. 6) at the end of the lactation period emphasizes the importance of functional corpora lutea to the inhibitory action of estrogen. The histological appearance of the mammary gland of the treated animals indicates that inhibition of lactation in the present experiments is correlated with proliferation of the alveolar tissue. The numerous small alveoli found in the mammary glands 10 to 12 days after the first injection of intact animals treated with estrogen and of ovariectomized rats treated with estrone and progesterone are lined with cells which suggest growth (cuboidal cells) and an abeyance of secretion (diminution or absence of fat globules). Fauvet (1941) observed similar histological changes in the mammary gland of ovariectomized rats treated simultaneously with estrogen and progesterone. It has been suggested that substances which induce mammary growth also bring about inhibition of lactation (Loeb, 1932; Nelson, 1934; Folley and Kon, 1937b; Nelson, Gaunt and Schweizer, 1943). Nelson (1937) has shown that estrone induces complete mammary development in the ovariectomized guinea pig. On the other hand, Nelson (1935) found that although estrone produces lobule proliferation in the ovariectomized rat it does not bring about complete development of the mammary glands. Our data from ovariectomized lactating rats indicate that estrone alone neither inhibits lactation nor stimulates growth of the alveoli, while a combination of estrone and progesterone does both. One of the combinations of estrone and progesterone (100γ and 1 mg.) selected for simultaneous injection before parturition is based on the observation of Scharf and

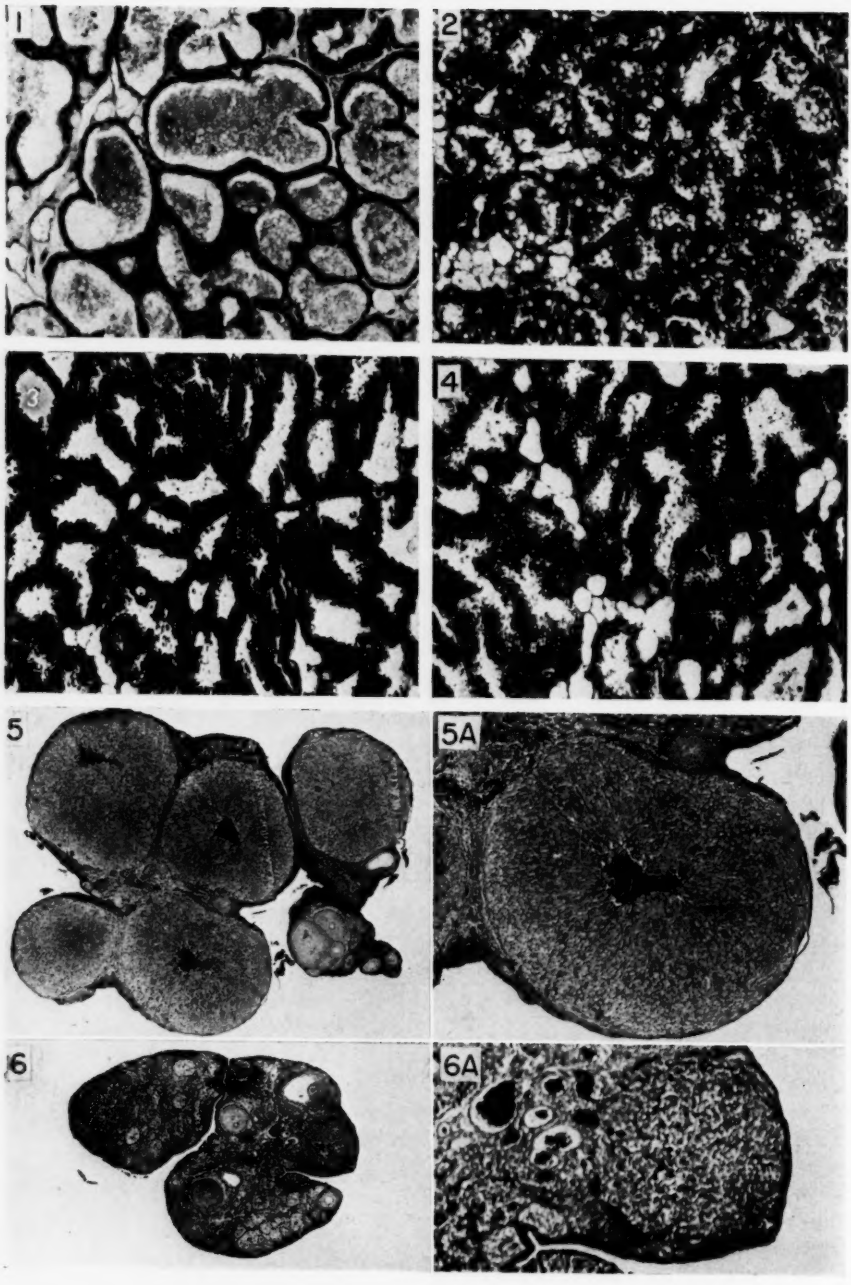

PLATE 1

Lyons (1941) that this combination showed the greatest alveolar development of the 6 combinations tried in immature male rabbits. The other combination (25γ of estrone to 2.5 mg. of progesterone) was used in order to make comparison with results already obtained by postpartal injections. The failure to prevent the initiation of lactation with either of these combinations of estrone and progesterone or with the hormones given separately suggests that removal of an inhibitory influence by reduction of the secretion of ovarian hormones in late pregnancy is not essential to the establishment of lactation provided maximal stimulation of lactogenic processes by nursing and removal of milk is obtained. However, it should be pointed out that it is not known whether the maximal inhibitory effects of these hormones have been attained in the present experiments. In fact, the greater effectiveness of the combination of 25γ of estrone and 2.5 mg. of progesterone in delaying the onset of lactation suggests that a combination of these hormones may be found which would completely prevent the initiation of lactation in the rat even with a maximal stimulus of nursing.

The assumption that inhibition of lactation in ovariectomized animals by estrogen requires dosages which exceed the normally occurring secretion is supported by observations on the rat. In our experiments doses of diethylstilbestrol dipropionate (1 mg. daily) large enough to depress lactation also brought about a marked loss of weight in the lactating animals. The depression of lactation in these rats is perhaps due to toxic effect on the lactating animal rather than to specific inhibition of lactation. Edelmann and Gaunt (1941) found that large doses of estrogen caused lactating rats to lose weight. Trentin and Turner (1941) found that the decrease of food intake in rats is

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EXPLANATION OF FIGURES ON PLATE 1

FIG. 1. Mammary gland removed on the 17th day after parturition from a rat ovariectomized on the day of parturition and injected daily with 100γ of estrone from the 2nd through the 16th day after delivery. ×140.

FIG. 2. Mammary gland removed on the 17th day after parturition from an intact rat injected daily with 100γ of estrone from the 2nd through the 16th day after delivery. ×140.

FIG. 3. Mammary gland removed on the 17th day after parturition from a rat ovariectomized on the day of parturition and injected daily with 100γ of estrone and 2.5 mg. of progesterone from the 2nd through the 16th day after delivery. ×140.

FIG. 4. Mammary gland removed on the 17th day after parturition from a rat ovariectomized on the day of parturition and injected daily with 25γ of estrone and 2.5 mg. of progesterone from the 2nd through the 16th day after delivery. ×140.

FIG. 5. Ovary (showing large corpora lutea) removed on the 17th day after parturition from a rat injected daily with 25γ of estrone from the 21st day of pregnancy through the 16th day after delivery. Compare with 6. ×10.

FIG. 5A. A higher magnification of a corpus luteum shown in figure 5. Compare with 6A. ×22.

FIG. 6. Ovary (showing atrophic corpora lutea) removed on the 17th day after parturition from a rat injected daily with 25γ of estrone from the 21st day of pregnancy through the 16th day after delivery. ×12.

FIG. 6A. A higher magnification of a corpus luteum shown in figure 6. ×33.

proportional to the increase of estradiol benzoate administration.

The extent of the influence of nursing or manipulation of the mammary gland and removal of milk on lactation is not known but it has been demonstrated that the mammary glands of non-nursing rats contain very little milk on the 7th day after parturition (Meites and Turner, 1942a). The prevention of lactation with estrone injection in pseudopregnant and in parturient rabbits by Smith and Smith (1933) was obtained in non-nursing animals and the removal of milk was accomplished manually. Our observation that injection of estrone before parturition in the intact rat does not prevent the initiation of lactation is contrary to the findings of Smith and Smith and in agreement with the observation of Edelmann and Gaunt (1941) in the rat. Nelson (1934) found that estrone given immediately after parturition prevented the initiation of lactation in the intact guinea pig. The failure to prevent the initiation of lactation by estrone administration in the rat suggests a species difference but the possibility that the lack of the stimulus of nursing may have been a factor in the experiments of Smith and Smith should be considered.

The failure of progesterone treatment, begun prepartally, to prevent the initiation of lactation opposes the view advanced by Meites and Turner (1942b) that progesterone might be responsible for the failure of abundant lactation to occur prior to parturition and indicates strongly that the initiation of lactation after parturition does not require the removal of an inhibitory action of progesterone. The failure of postpartal injection of progesterone to inhibit established lactation is in agreement with the findings of Anselmino and Hoffmann (1936) and Folley and Kon (1937a).

SUMMARY

Observations were made on the effects of prepartal and postpartal administration of estrogens, of progesterone and of combinations of estrone and progesterone on lactation in intact and in ovariectomized rats, using the growth rate of nursing young to indicate the rate of milk secretion.

Daily (200γ) treatment with estrone or diethylstilbestrol dipropionate begun on the second day of lactation does not inhibit milk secretion in ovariectomized rats while moderate daily (5γ and 10γ) injection of estrone inhibits lactation in intact rats, the inhibition appearing 10 to 12 days after the beginning of treatment. The inhibition of lactation induced by treatment with still larger dosage (1 mg.) of diethylstilbestrol dipropionate both in intact and in ovariectomized rats was accompanied by marked loss of weight of the mother and is interpreted as a toxic effect. Prepartal injection of estrone does not prevent the initiation of lactation either in ovariectomized (25γ) or in intact (25γ and 100γ) rats but does produce a delayed depression in intact animals.

Daily treatment with progesterone begun prepartally (2.5 mg.) or postpartally (5 mg.) neither prevents nor inhibits lactation.

Simultaneous injection of estrone and progesterone begun prepartally or postpartally does not prevent lactation but it does inhibit established lactation after 10 to 12 days of treatment.

The marked proliferation of the mammary glands showing inhibition of lactation suggests that stimulation of mammary growth by combined action of estrone and progesterone plays a role in the inhibition of milk secretion in the rat.

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CHOLINERGIC AND ADRENERGIC COMPONENTS IN THE NEUROHUMORAL CONTROL OF THE RELEASE OF LH IN THE RABBIT

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INTRODUCTION

The nervous system, in species like the rabbit which do not ovulate spontaneously, controls ovulation by stimulating the anterior lobe of the hypophysis to release lutenizing hormone; LH in turn (alone or in combination with FSH) activates maturation and rupture of the ovarian follicles (Hisaw, 1947). The nature of the mechanism by which the central nervous system exerts its influence on the hypophysis has been the subject of numerous investigations including those of Markee, Sawyer, and Hollinshead (1946–1948). Electrical stimulation experiments by these authors (1946) gave evidence that hypothalamic control of the anterior lobe, which almost certainly involves the pituitary stalk (Brooks, 1940), is not exerted via nerve fibers ending in the pars distalis. The results indicated, rather, that a humoral link, such as the hypophyseal portal system (Wislocki and King, 1936; Green and Harris, 1947), might transport a chemical mediator capable of stimulating the electrically-inexcitable gland cells. Attempts to stimulate LH release by intravascular administration of the neurochemical agents, acetylcholine and adrenaline, led to uniformly negative results (Markee, Sawyer and Hollinshead, 1947, 1948). When adrenaline was introduced directly to the adenohypophysis, however, ovulation was induced in a significant number of cases —5 out of 10 with the optimal concentration (1/1000). Acetylcholine was ineffective even when applied in this direct manner. The presence of an adrenergic component in the natural secretion-stimulus was confirmed (Sawyer, Markee and Hollinshead, 1947) by blocking copulation induced ovulation with Dibenamine, which is a potent adrenolytic agent.

Inasmuch as direct intrahypophyseal administration of acetylcholine failed to stimulate release of LH in the rabbit, as it had also failed in the constant-estrous guinea pig (Dey, 1943), no attempt was made at the time of the Dibenamine study to block the coital stimulus with anti-cholinergic agents such as atropine. Taubenhaus and Soskin (1941) had reported, however, that direct application of acetyl-

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choline to the rat hypophysis results in liberation of luteotrophic hormone. Although the normal hypophyseal control of the corpus luteum in the rabbit appears to be an indirect one (Robson, 1938), it depends upon the integrity of the pituitary stalk (Westman and Jacobsohn, 1940) and might involve a cholinergic mediator. Therefore, to ascertain whether it might interfere with luteal function, we injected atropine intravenously into female rabbits within a minute after copulation. Interestingly enough, ovulation was blocked in a significant number of cases.

The discovery that atropine blocks copulation-induced release of LH implies that the natural neurogenic stimulus includes a cholinergic as well as an adrenergic component. This inference led to a careful study of the time relationships between the atropine-blocked and Dibenamine-blocked components in order to determine whether they function simultaneously or sequentially. In the earlier Dibenamine study the presence of ruptured or hemorrhagic ovarian follicles as observed at laparotomy was employed as the sole criterion of pituitary stimulation. In order to study the effects of partial activation, the present work includes an histological examination of non-ovulating as well as ovulated follicles.

The results of the present study indicate that a cholinergic component of the neurogenic[1] stimulus for the release of LH in the rabbit precedes the adrenergic component and may be responsible for it. Luteal function is not inhibited by either adrenolytic or anti-cholinergic agents, at least not in dosages which block the release of LH. Most of the ovarian follicles whose ovulation has been blocked by atropine or Dibenamine give histological evidence of partial activation. This partial activation includes various degrees of maturation of the ovum, corona formation, secretion of secondary liquor folliculi, and an atypical variety of atresia in which the ovum of a large follicle precedes the granulosa in degeneration. Part of the results of the present study have already appeared in abstract form (Sawyer, Markee and Townsend, 1948).

MATERIALS AND METHODS

Ninety-four female rabbits were employed in the present investigation. Many were used several times so that the total number of experiments exceeded 160. The average weight was 3.2 kg. and only 2 animals weighed less than 2.4 kg. Most of the rabbits were segregated for weeks before mating though some were used after a few days isolation. Among the latter group, seven were found at laparotomy, 48 hours after mating, to have been pregnant a week or more. None of them ovulated in response to the experimental mating; these cases were excluded from the results of the pharmacological blocking experiments. Also excluded were cases of rabbits whose ovaries were

[1] The term *neurogenic* as used in this paper indicates that the nervous system is involved, but we do not intend it to imply that the stimulus is transmitted by nerve fibers ending in the adenohypophysis.

found to be immature and which failed to ovulate either during the attempted blocking experiment or at subsequent mating two or more weeks later when no drug was employed. It has been shown many times that mating in immature or pregnant females does not result in ovulation (Hammond and Marshall, 1925).

Solutions of atropine and Dibenamine were freshly prepared from the salts[2] on the day in which they were to be used. Dibenamine was dissolved first in absolute alcohol (100 mg./ml.) and then diluted to 10 mg./ml. with unbuffered Ringer-Locke solution. The effective dose, 30 mg./kg., thus required a total volume of 10 ml. for a 3.3 kg. rabbit, and the total solution per animal was injected in about 45 seconds. This dosage was above the minimum lethal dose as observed earlier (Sawyer *et al.*, 1947); in the present work 5 of a total of 41 rabbits which received 30 mg. Dibenamine per kg. expired during the subsequent 16 hours. Atropine was dissolved directly in Ringer-Locke solution and concentrations of 10 mg./ml., 30 mg./ml. and 60 mg./ml. were employed. In preliminary experiments, the results of which will be presented below, dosages of 1, 5, 10, 30, 50, 75 and 100 mg./kg. were injected to determine effective and lethal dosages.

In most of the mating experiments the service of a single vasectomized male was engaged. In a typical experiment the female was placed in a large cage with the male. At the instant after copulation when the male fell off, a stop watch was started and the female hastily removed to an injection box. Injection of the drug into the marginal ear vein was begun immediately or at definite intervals (15 sec., 30 sec., 5 min.) post coitum, and the time at which injection was terminated was also noted. "Rapid" injection experiments eliminated the transferral of the female; one person held her at the termination of coitus while another injected concentrated atropine (60 mg./ml.) as rapidly as possible; only the time of completed injection was recorded in this series.

The ovaries were examined at laparotomy or autopsy usually at 48 hours though occasionally at 24 or 72 hours, after copulation. Unilateral ovariectomy was often performed to obtain histological material for comparison with the remaining ovary, which was to be fixed at autopsy at a later time, under different experimental conditions. Tissues were fixed in Zenker's fluid, sectioned at 10 μ, and stained with Harris' hematoxylin and Everett's (1943) modification of Mallory's tri-acid stain.

The integrity of luteal function was tested by an histological examination of uteri and corpora lutea of animals autopsied at 9 to 14 days after mating, and by the histological confirmation of deciduomata established by threading the uterus at five days post coitum and waiting five or six more days before autopsy (Brouha, 1934).

RESULTS

Establishing an optimal dose of atropine. The two rabbits which received atropine in dosages of 75 and 100 mg./kg. rapidly expired, while they lay on their sides and gasped, and a few apparently

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[2] Atropine sulfate USP XIII (Mallinkrodt) and Dibenamine hydrochloride were used. We are grateful to Dr. William Gump of Givaudan-Delawanna, Inc., for a generous supply of Dibenamine.

spontaneous fasciculations occurred in their otherwise hypotonic muscles. Of 7 animals injected with 50 mg./kg., 2 showed little effect beyond pupillary dilation, 2 required artificial respiration to recover, and 3 died in spite of attempts to resuscitate them. Thus 50 mg./kg. is in the neighborhood of the LD_{50}. Preliminary attempts with 30 mg./kg. led to no fatalities but did induce "spontaneous" muscular activity and respiratory embarrassment. This dosage, which was adopted for use in subsequent experiments, has been found to be above the MLD for it was lethal to at least 3 animals of 78 receiving it in the blocking experiments. Lower dosages produced no apparent effect except pupillary dilation, which was induced by as little as 1 mg./kg.

Blocking experiments with atropine. The results of atropine-blocking experiments which were conducted independently of Dibenamine experiments are summarized in Table 1. Of 17 rabbits receiving their total dose of 30 mg./kg. within a minute after the termination of

TABLE 1. EFFECT OF ATROPINE SULFATE (30 MG./KG. INTRAVENOUS) ON THE ACTIVATION OF THE RABBIT ADENOHYPOPHYSIS
(Laparotomy at 48 hrs. post coitum)

Number of animals	Time injection was terminated (relative to copulation)	Ovulated (hypophysis activated)	Failed to ovulate (stimulus blocked from reaching hypophysis)	Percentage blocked by atropine
11 {3	2, 4, 9 days before		3	
11 {8	1, 2, 2, 5, 8, 8, 9, 12 d. before	8		27
17	1 minute after*	8	9	53
12	5½ minutes after	11	1	8
13	10–40 seconds after*	4	9	69

* These injections were *begun* as soon as possible—certainly less than 15 seconds after copulation (cf. table 2).

coitus, 9 were found at laparotomy 48 hours later to have failed to ovulate. This proportion, 9/17, assumes a high degree of significance in view of the fact that somewhat less than one animal (non-pregnant and apparently mature) in ten fails to ovulate in response to a single mating followed by no drug (unpublished observations). The probability that 9 out of 17 would have failed without the atropine is about one chance in a hundred thousand ($P = 0.00001$).

When atropine was withheld for 5 minutes after copulation, however, 11 out of 12 animals ovulated, a figure almost identical to the chance probability without atropine. In these two series a concentration of 30 mg./ml. was employed: the rabbits received 1 ml. solution per kg. weight.

A more rapid injection series, in which a concentration of 60 mg. atropine per ml. was used and the total (30 mg./kg.) dose was in-

jected within 10 to 40 seconds post coitum, contained a higher proportion of failures to ovulate (9/13 or 69%). The four rabbits which did ovulate in this series were observed to be especially "hot." They raised their pelves immediately on being mounted while the others of the series required several seconds or even minutes of the male's efforts before they responded.

No animal which received 30 mg. atropine per kg. would mate later on the same day—the drug presumably threw her out of heat. Of 11 female rabbits that were injected with this dosage of atropine and mated as soon as they returned to heat within twelve days after injection, 3 failed to ovulate. This is not very significantly different from the chance ratio: $P = 0.07$.

Comparative blocking experiments: Atropine vs. Dibenamine. Neither atropine nor Dibenamine will block ovulation if the injection is delayed many minutes after copulation: almost 100% of the cases ovulated after delayed injections of either drug (see above and Sawyer, Markee and Hollinshead, 1947). The proportion in which pituitary activation is blocked by atropine within one minute post coitum (9/17 or 53%) is low when compared with the results of the one-minute Dibenamine experiments (16/19 or 84%). The fact that an increased percentage of atropine blockage (69%) is achieved by increasing the rapidity of the injection, suggests that the inferiority of atropine to Dibenamine as a blocking agent may rest in temporal factors: the atropine-blocked phenomena may reach completion before the Dibenamine-blocked activities cease.

Before attempting to differentiate more accurately the temporal relationships of the blocking effects, it was desirable to demonstrate the rapidity with which intravenous atropine and Dibenamine reach and evoke a response from effector organs near the hypophysis. The fact that the iris reacts to both drugs, pupillary dilatation or constriction following atropine or Dibenamine, respectively, makes the iris an ideal indicator for such a comparison. Three rabbits were injected with 30 mg. Dibenamine per kg.; the solution was made up as 10 mg./ml., and the injection was delivered at such a speed as to terminate at 45 seconds. The pupils started to constrict, on the average, at 25 seconds, and the constriction was complete at 35 seconds from the beginning of injection. Four rabbits received injections of atropine at identical concentrations, dosages and speeds, with the results that pupillary dilatation began at 21 seconds and was complete at 32 seconds. Thus, it is possible to state that atropine arrives at the base of the brain in sufficient concentrations to induce anti-cholinergic effects at least as rapidly as Dibenamine does to exert its adrenolytic effects. Therefore, Dibenamine cannot be considered the better blocking agent merely by virtue of superior capacity to survive the intravascular journey. It should be noted, in passing, that rapid injections of atropine (dosage, 30 mg./kg.; concentration, 60 mg./ml.; 3

TABLE 2. COMPARATIVE EFFECTS OF ATROPINE AND DIBENAMINE ON THE ACTIVATION OF THE RABBIT ADENOHYPOPHYSIS FOLLOWING COPULATION

Injection time (seconds after copulation)		Drug	Number of Animals	Ovulated	Failed to ovulate	Percentage blocked by drug
Begun	Ended					
30	60–90	Atropine	10	9	1	10
		Dibenamine	10	5	5	50
15	60	Atropine	20	17	3	15
		Dibenamine	13	3	10	77

rabbits) initiated pupillary dilation at less than 15 sec. and dilation was completely by 20 sec. Such rapid injections of Dibenamine are not feasible because they are lethal; furthermore high concentrations of Dibenamine are insoluble in saline even after preliminary solution in alcohol.

To demonstrate more adequately whether the atropine-blocked component of the neurogenic stimulus at copulation actually precedes in time the Dibenamine-blocked component, an attempt was made to find an injection time at which atropine might be completely ineffective and Dibenamine completely effective. The results of these attempts are presented in Tables 2 and 3. They are 4 entirely new series, the experiments of which were initiated after the completion of Table

TABLE 3. DEGREES OF COPULATION-INDUCED PITUITARY ACTIVATION ALLOWED BY DIBENAMINE AND ATROPINE INJECTED AT SIMILAR TIMES IN INDIVIDUAL RABBITS

(Dibenamine, 30 mg./kg., injections started at 15 sec. or later after copulation. Atropine, 30 mg./kg., injections started at 15 sec. or earlier after copulation).

Rabbit Number	Wgt. Kgms.	Drug	Date	Inject. time, sec.		Results Ovary, gross and histological appearance
				Begun	Ended	
352	3.0	Diben.	4/29	30	60	Clear foll., no sections
		Atrop.	5/4	15	60	Rupt.+hem. foll.; well luteinized
346	3.0	Diben.	5/24	30	90	Hyperemic foll.; activation atresia = #384
		Atrop.	5/4	15	60	9 Rupt. foll.; no sections
318	3.1	Diben.	5/29	15	70	Hyperemic foll.; slight activation = #333
		Atrop.	5/4	15	60	9 Rupt.+hem. foll.; no sections
356	3.1	Diben.	5/6	15	60	4 Rupt. foll.; no sections
		Diben.	6/12	15	60	10 Rupt. foll; well luteinized
		Atrop.	5/24	15	60	10 Rupt. foll; well luteinized
372	2.4	Diben.	6/8	15	60	Clear foll.; v. slight activation = #372
		Atrop.	5/11	—	17	4 Rupt.+hem. foll.; no sections
		Atrop.	6/19	15	60	9 Rupt.+hem. foll.; well luteinized
383	3.4	Diben.	5/24	15	60	Hyperemic foll.; activation atresia, Fig. 15
		Atrop.	5/29	15	60	Hyperemic foll.; slight activation, Fig. 5
387	2.9	Diben.	5/24	20	75	Clear foll.; v. slight activation = #372
		Atrop.	6/2	15	60	11 Rupt. Foll.; well luteinized
384	2.9	Diben.	6/12	15	60	Hyperemic foll.; activation atresia Fig. 12
		Atrop.	5/24	15	60	11 Rupt. foll.; well luteinized
333	3.3	Diben.	6/22	30	60	Clear foll.; slight activation, Fig. 4
		Atrop.	5/29	15	55	10 Rupt. foll.; well luteinized, Fig. 16
393	3.9	Diben.	6/2	15	60	Clear foll.; moderate activation, Fig. 6
		Atrop.	6/8	15	60	10 Rupt. foll.; well luteinized

1. It was found that injections beginning at 30 sec. post coitum were 90% ineffective with atropine but only 50% effective with Dibenamine. Actually this latter proportion, 5/10, represents a highly significant degree of blocking effectiveness; with 1/10 as the chance failure the probability that 5/10 will fail without the drug is less than one chance in six hundred ($P = 0.0015$). Nevertheless, a second series, with injections beginning at 15 sec., was undertaken. Under these time relationships atropine was 85% ineffective and Dibenamine was 77% effective. The atropine ratio of 3/20 is still not significantly different from the chance probability ($P > 0.1$), but the Dibenamine ratio of 10/13 would occur by chance less than one time in ten million. All four of the atropine-treated animals which failed to ovulate, as well as most of the Dibenamine-blocked rabbits, gave histological evidence of partial pituitary-activation as will be mentioned below.

An even more significant comparison is presented in Table 3, which employs some of the 15-sec. results of the same animals as those summarized in Table 2. Ten rabbits received, on separate occasions, both atropine (at 15 sec. or earlier) and Dibenamine (at 15 sec. or later). Of these, one rabbit ovulated in spite of the injection of either drug (at exactly 15 sec.); one failed to ovulate following either (at exactly 15 sec.); the other eight ovulated after atropine (at 15 sec. or earlier . . . e.g. #372) and were blocked by Dibenamine (at 15 sec. or later—up to 30 sec.). The ovaries of most of these animals were studied histologically, and considerable evidence of the partial activation of follicles is summarized in Table 3. This phenomenon of partial activation will be considered in detail in the next section.

Partial activation of follicles in cases of blocked ovulation. In the rabbit healthy large ovarian follicles do not undergo the maturation process without the mating stimulus. The stages of normal post-coital maturation of the follicle and ovum have been often described (Pincus and Enzmann, 1935, 1937; Waterman, 1943) and will be only briefly outlined here. They include migration of the nucleus of the ovum to the periphery and its flattening there, formation of tetrads, loss of the nuclear membrane, formation of the spindle for and extrusion of, the first polar body, and formation of the spindle for the second polar body. The second polar body is not normally extruded until after ovulation, or rupture of the follicle, which occurs between 10 and 14 hours after mating. While the ovum is maturing, preovulatory changes are occurring in the follicle, including secretion of the secondary follicular liquor (Robinson, 1918). This secretion is usually first seen in and around the cumulus, and as the cells of the cumulus loosen the corona radiata becomes more apparent. There is often a sharp line of demarcation between the finely granular secondary secretion and the coarser primary liquor; Dawson and Friedgood (1940) have described in the cat a "streaming" of the secondary liquor into the primary, which breaks up the boundary that separates them.

There is little doubt that the normal maturation changes are induced by pituitary gonadotrophic hormone released in response to the copulation stimulus. Apparently the threshold for maturation is less than that for complete ovulation: Hinsey and Markee (1933a) found that small hypophysectomized rabbits which would not ovulate in response to prolan injection nevertheless underwent maturation to the stage of second polar spindle formation. Furthermore, Pincus and Enzmann (1935) induced maturation in rabbits with as little as one fourth the minimal ovulating dose of gonadotrophic preparation.

Atresia of ovarian follicles may occur at any stage in their development. In typical atresia of small follicles the primary degenerative changes occur in the egg, and these are followed by atrophy of the granulosa. In atresia of medium and large follicles, on the other hand, degeneration of the granulosa is the primary factor, and it may be quite complete before the ovum appears to suffer (Asami, 1920; Pincus and Enzmann, 1937). In typical atresia of large follicles maturation changes in the ovum may follow pronounced degeneration of the granulosa including loosening of the corona. Pincus and Enzmann have suggested that the stimulus to such maturation is some nutritional change in the ovum.

Our histological preparations of the ovaries of rabbits in which ovulation was blocked by drugs include all 4 of the atropine-blocked cases presented in Tables 2 and 3, and 12 of the 15 Dibenamine-blocked cases found in these tables. We also have sections of the ovaries of 7 rabbits which ovulated in spite of Dibenamine and 15 animals whose ovulation was not blocked by atropine.

All four of the atropine-blocked cases gave histological evidence of partial activation of their follicles and, by implication, their hypophyses. The same is true of most of the Dibenamine-blocked cases. This evidence is presented in Fig. 1 to 15 and in the following paragraphs. References are made in Table 3 to the conditions in individual animals and cross-references indicate the appropriate figures in which those conditions are illustrated.

Injection of atropine or Dibenamine into an unmated estrous rabbit produces no follicular activation that is apparent at autopsy, 48 hours later (Figs. 1 and 2). Three of the Dibenamine-blocked animals give little evidence of activation (Fig. 3). Two of the "15-second" Dibenamine and one of the "15-second" atropine animals reveal ova and follicles at 48 hours which are characteristic of the normally stimulated ovary about 4 hours after mating (Figs. 4 and 5). Four other mated and Dibenamine-treated females show fairly typical maturation stages that are characteristic of the normal animal 7 to 10 hours after mating (Figs. 6–9).

The other 3 atropine-blocked rabbits (Table 2) and 9 of the Dibenamine-blocked specimens reveal at 48 hours an atypical or activation-type of follicular atresia. A low-powered photomicrographic comparison between typical and atypical atresias is seen in Figs. 10

and 11. The former, as already mentioned, is characterized in large follicles by a primary degeneration of the granulosa. Atypical or activation-atresia (Figs. 11, 12, 14, 15) is characterized by degeneration of the ovum in an intact follicle; mitotic figures are usually found in the granulosa; secretion of secondary liquor and organization of the corona testify to the activation. The degenerating ova usually stain metachromatically; they may or may not contain vesicular nuclei, and they are often apparently invaded by cellular elements which are stained by aniline blue (Figs. 12 and 14). We have seen similar activation-atresia in follicles which failed to rupture in a normal unblocked ovulation but not in non-stimulated ovaries.

Two experimental animals have revealed typical, though retarded, activation pictures at 48 hours following one treatment and atypical atresia following another. Rabbit #380 showed maturation to polar body formation when atropine was injected at 10 sec. post coitum (Fig. 13), and on another occasion activation-atresia when atropine was administered at 15 sec. post coitum (Fig. 14). Rabbit #383 had atypically atretic follicles following 15 sec. Dibenamine blockage (Fig. 15) and slight typical maturation when atropine was injected at 15 sec. post coitum (Fig. 5).

Histological preparations of follicles which ovulated in spite of 15-second atropine injections (Fig. 16) and 15-second Dibenamine injections (Fig. 17) all showed considerable luteinization at fixation 48 hours post coitum. The Dibenamine-treated specimens appeared to be fully as far advanced in the process as the atropine-injected cases.

Blocking agents and luteal function. Ten rabbits were utilized to

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EXPLANATION OF FIGURES ON PLATE 1

Apart from figures 1 and 2, all of the follicles and ova pictured in plates 1 and 2 are from ovaries fixed at laparotomy or autopsy at about 48 hrs. (range 43 to 52 hrs.) after mating with a vasectomized buck.

FIG. 1. Section through a resting graafian follicle of a rabbit ovary fixed at 48 hours after an intravenous injection of Dibenamine. Note the absence both of a corona and of secondary follicular liquor. ×40.

FIG. 2. A higher magnification of the ovum in figure 1, showing the vesicular nature of the cytoplasm, the intact nuclear membrane and a large nucleolus. ×210.

FIG. 3. Section of an ovarian follicle from a rabbit (#372) which received intravenous Dibenamine at 15 seconds post coitum. There is evidence of very slight activation —a little secondary liquor in and around the cumulus and a granulation of the cytoplasm of the ovum. ×60.

FIG. 4. Definite signs of follicular activation including flattening of the nucleus at the periphery of the ovum, corona formation and an observable contrast between the finely granular secondary liquor and the coarser primary liquor. The rabbit (#333) received Dibenamine at 15 seconds post coitum. ×75.

FIG. 5. Ovum from a rabbit (#383) which received atropine at 15 seconds post coitum. The degree of activation is about equal to that in figure 4; under normal conditions this stage would be reached at about 4 hours after copulation. Cf. fig. 15. ×210.

FIG. 6. Evidence of further activation is seen in this follicle from a rabbit (#393) which received Dibenamine at 15 seconds post coitum. There is a sharp boundary, Robinson's "membrane," between primary and secondary liquors, and the spindle for

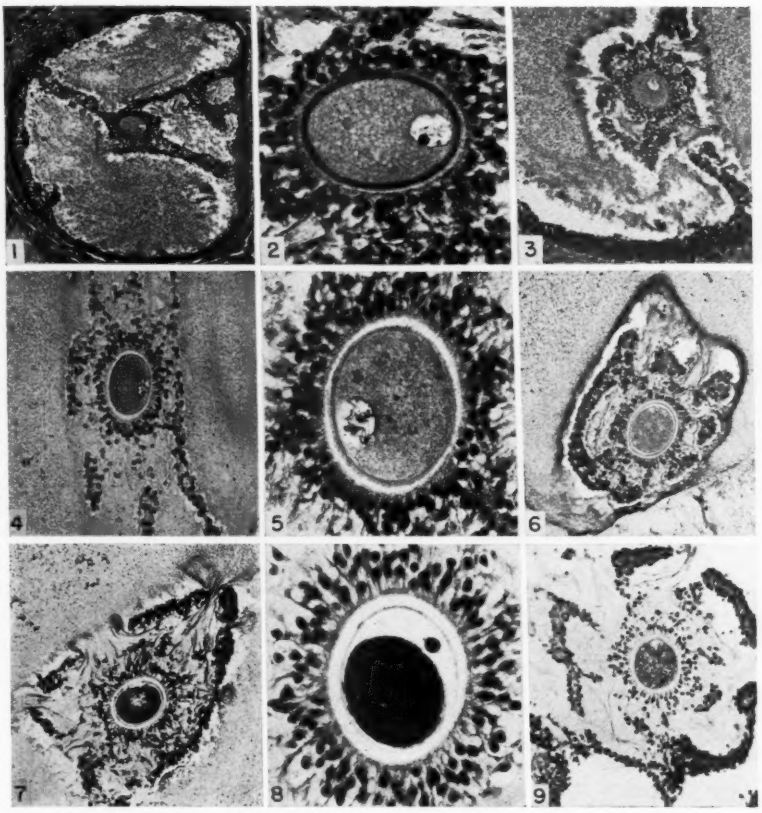

PLATE 1

extrusion of the first polar body is seen as the shadow at the lower left pole of the ovum. ×75.

FIG. 7. Section of a follicle which illustrates "streaming" of the secondary liquor into the primary. The zona pellucida contains a first polar body above the ovum. The rabbit (#379) received Dibenamine at 10 seconds post coitum. The follicle, one of four containing polar bodies in the right ovary of rabbit #379 ,would probably not have ruptured, since the left ovary, fixed eleven days later, contained no recent corpora lutea. ×75.

FIG. 8. Ovum and first polar body from an ovary of rabbit #376, which received Dibenamine at 30 second post coitum. The stage represented by figures 7 and 8 is normally reached at 8 to 10 hours after mating. ×210.

FIG. 9. Section of follicle in which the ovum contains the second polar spindle: the first polar body is not in the plane of section. Note the well developed corona and "streaming" of secondary liquor. It is at this stage of maturation that ovulation ordinarily occurs. The rabbit (#348) received Dibenamine at 15 seconds post coitum. ×75.

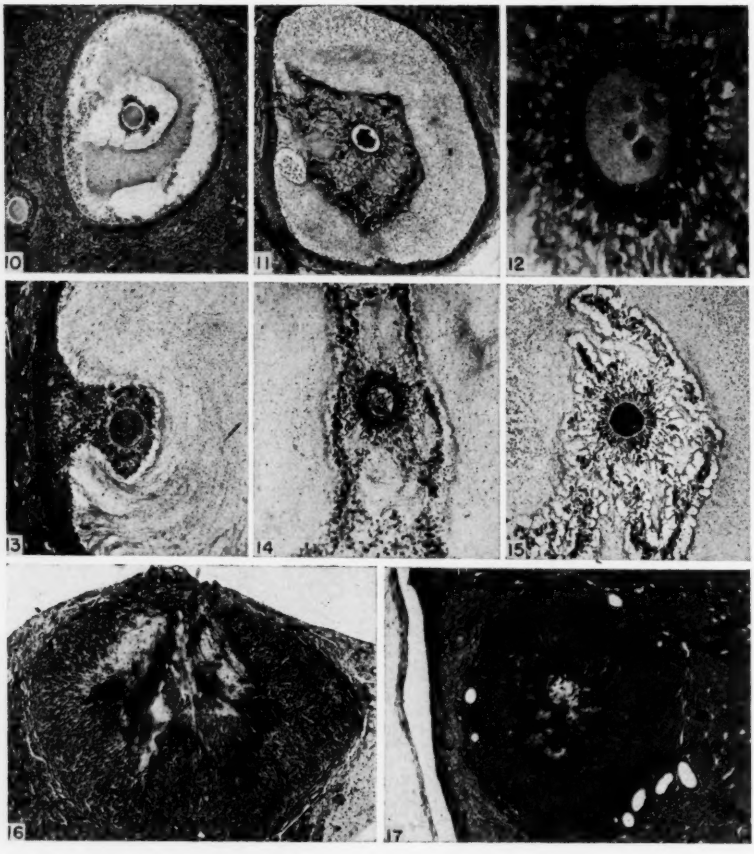

PLATE 2

Explanation of figures

FIG. 10. Large follicle undergoing typical atresia. The granulosa has almost completely degenerated while the ovum, although lacking a vesicular nucleus, is still intact. This follicle (maximum diameter, 0.97 mm.) is only slightly smaller than the resting follicle (maximum diameter, 0.92 mm.) in fig. 1. ×35.

FIG. 11. Section through a follicle undergoing atypical or activation-atresia. The ovum is degenerate while the granulosa is intact. Activation by LH is indicated by the size of the follicle (cf. fig. 10), the definite corona, and the secondary liquor folliculi which is separated from the primary liquor by a distinct "membrane." This follicle and the preceding one were both from an ovary of rabbit #374, which was the only female that failed to ovulate when injected with atropine at 30 seconds post coitum (vide Table 2). ×35.

FIG. 12. Ovum from an atypically atretic follicle. The animal, #384, received Dibenamine at 15 seconds post coitum. Note the distinct corona and the elements (phagocytes?) within the ovum. ×185.

FIG. 13. Section showing ovum with polar body but otherwise slight activation—little secretion of secondary liquor. The follicle was from the right ovary of rabbit #380 after treatment with atropine at 10 seconds post coitum (cf. fig. 14). ×65.

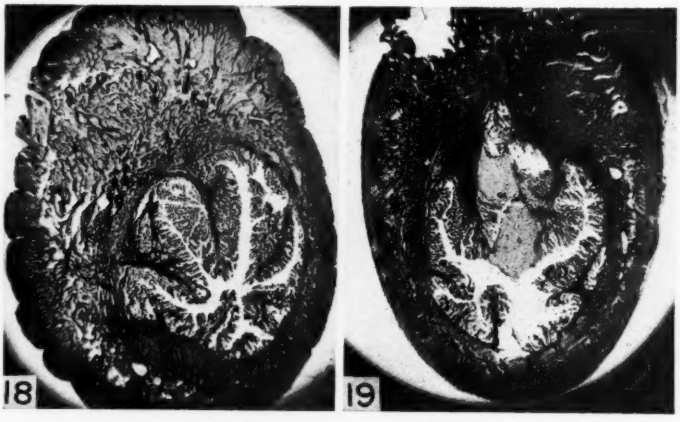

PLATE 3

Explanation of figures

FIG. 18. Cross section of a rabbit uterus fixed 9 days after a sterile mating which was followed within a minute by an intravenous injection of atropine. Note the progestational proliferation of the glands. The corpora lutea looked normal for 9 days of pseudopregnancy. ×8.

FIG. 19. Uterus with deciduomata 11 days after mating and 6 days after threading. Atropine was injected at 5 minutes post coitum and daily thereafter until autopsy. ×8.

determine whether atropine or Dibenamine interfered with the secretion of progesterone. The animals were mated with a vasectomized buck and 6 were injected with atropine and 2 with Dibenamine (30 mg./kg. in each case) at 1 or 5 minutes post coitum; two others served as controls. Two of the 5-minute atropine-treated females also received daily injections of 30 mg. atropine per kg. until the day of autopsy. Laparotomy revealed that all 10 had ovulated. At a second laparotomy 5 days post coitum, the uteri of 7 animals (1 Dibenamine-treated, 4 atropine-treated and 2 controls) were threaded to induce formation of deciduomata. The 3 rabbits whose uteri were not threaded (1 Dibenamine-treated, 2 atropine-treated) were autopsied at 9 days post coitum. Their uteri, in histological sections, all showed progestational proliferation of the glands (Fig. 18). The 7 uterus-

FIG. 14. Activation-atresia in a follicle of the left ovary of rabbit #380 after atropine injection at 15 seconds post coitum (cf. fig. 13). Definite activation is indicated by the secondary liquor and the corona. The elements within the ovum are perhaps phagocytes; they are probably not blastomeres for a vesicular nucleus is still present in a similarly "invaded" ovum of another follicle in the same ovary. ×65.

FIG. 15. Activation atresia after Dibenamine treatment at 15 seconds post coitum (rabbit #383) for comparison with figure 5. ×65.

FIG. 16. Ruptured follicle at 48 hours after mating. The rabbit (#333) received atropine at 15 seconds post coitum. Cf. figures 4 and 17. ×35.

FIG. 17. Ruptured follicle at 48 hours; Dibenamine at 15 seconds post coitum. The degree of luteinization is approximately equal to that in the atropine-treated case, figure 16. ×35.

threaded animals were autopsied at 5 or 6 days after the threading. Histological preparations revealed an unmistakable deciduoma in at least one uterine horn of each rabbit. The deciduomata were small in two of the atropine-treated females but were extensive in the 2 animals which received the daily atropine injections (Fig. 19). At autopsy all of the corpora lutea looked normal, and histological sections from 2 of the "5-minute" atropine animals showed no apparent morphological changes from the control. Thus atropine and Dibenamine may be said to exert no overt influence on luteal function after the stimulus for LH release has reached the adenohypophysis.

DISCUSSION

Earlier attempts to block ovulation in the rabbit with atropine differ from the present experiments in two important details: in the former the dosages were much smaller and were administered just prior to copulation. Foster, Haney and Hisaw (1934) reported that atropine in doses up to $2\frac{1}{2}$ mg./kg. injected between 5 and 20 minutes before copulation inhibited ovulation in 7 rabbits. However, Makepeace (1938), repeating their experiments with estrous (post-partum) rabbits, reported that 11 out of 13 ovulated in spite of atropine doses up to 6 mg./kg. Since the experimental conditions in the present work differed so extensively from those in the earlier investigations, the positive results cannot be considered a direct confirmation of the findings of Foster, Haney and Hisaw. The high dosage (30 mg./kg.) employed in the present experiments could not be administered prior to copulation for it threw the animals out of heat for one or more days; Makepeace reported a similar effect in most of the animals injected with 6 mg. atropine per kg. In defense of the possible criticism that our higher dose blocked ovulation by an unspecific debilitating effect on the animals, we need merely point out that a few seconds' delay in injection allowed ovulation to proceed in an unimpaired manner. Actually some of the rabbits in preliminary experiments withstood 50 mg./kg. without noticeable effects beyond pupillary dilation; their sera probably contained high complements of atropinesterase, an enzyme which is found in one out of four rabbits (Glick and Glaubach, 1941). Due to the probable presence of atropinesterase in some of the experimental animals, the 30 mg./kg. dose may have been too weak in certain cases, but the improvement in the percentage of blocking by increasing the rapidity of injection indicates that the time factor may well account for the failures.

Taubenhaus and Soskin (1941) reported that in the rat preliminary treatment of the hypophysis with atropine blocked the pseudopregnancy which usually follows genital stimulation. This they attributed to an inhibition of the release of "luteinizing" hormone but luteotrophic hormone (lactogen) has subsequently been shown to control luteal function in the rat (Astwood, 1941; Evans *et al.*, 1941).

The experiments of Robson (1938) suggest that in the rabbit the hypophyseal control of the secretion of the corpus luteum is an indirect one, but a neural element in such control is indicated by corpus luteum degeneration in rabbits whose pituitary stalks have been cut (Westman and Jacobsohn, 1940). In the present experiments neither atropine nor Dibenamine interfered with luteal function. This could mean, however, merely that single or daily injections of drugs, while capable of blocking the temporary neurogenic stimuli for a rapid surge of gonadotrophic hormone, are of insufficient duration to block chronic neurogenic stimuli as completely as does section of the hypophyseal stalk.

The positive results on cholinergic stimulation of luteotrophic hormone in the rat (Taubenhaus and Soskin, *op. cit.*) and on adrenergic stimulation of LH release in the rabbit (Markee, Sawyer and Hollinshead, 1948) suggest either that various pituitary hormones require different neural mediators or that various species utilize different mediators in their over-all neural control of the adenohypophysis. That the latter, at least in respect to the liberation of LH, is not true has recently been demonstrated by Sawyer, Everett and Markee (1948) and by Everett, Sawyer and Markee (1948), who found that in the rat as in the rabbit the release of LH is blocked by Dibenamine. More recent experiments of the same investigators (unpublished) indicate that the cholinergic-adrenergic mechanism proposed here for the rabbit may also hold true in the stimulation of release of rat LH.

The present work emphasizes the speed with which the coital stimulus reaches the hypophysis. Prior to the appearance of the earlier Dibenamine results (Sawyer, Markee and Hollinshead, 1947) it was thought that the stimulus required up to half an hour to reach the gland. Westman and Jacobsohn (1940) reported that sectioning the stalk as late as 35 minutes post coitum blocks ovulation, and none of their animals whose stalks were cut less than 25 minutes post coitum ovulated. From our more recent knowledge it seems obvious that Westman and Jacobsohn's operation must have interfered with the output of LH rather than the reception of the particular coital stimulus which they were trying to block. Our earlier Dibenamine experiments showed that the coital stimulus reaches the hypophysis in less than 3 minutes. The present Dibenamine results, assuming that a blocking dose reaches the hypophysis within 25 seconds after the start of injection, indicate that in half of the animals sufficient stimulus for the release of an ovulating surge of LH reaches the hypophysis within 55 seconds post coitum. The stimulus for liberation of sufficient LH to activate the follicles of most ovaries and to ovulate some has reached the pituitary within 40 seconds post coitum. The time relationships of the completion of the atropine-blocked component are still less, as some of the "rapid-injection" animals ovu-

lated in spite of a blocking dose which induced pupillary dilation within 25 seconds post coitum.

The neurogenic stimulation of the release of LH is not an all-or-nothing phenomenon since partial activation results in the liberation of reduced amounts of hormone. Such a mechanism probably explains the delayed ovulations observed after artificial stimulation of the hypophysis (Marshall, 1942); some of those ovulations occurred as much as 48 hours after stimulation. In the present work, with laparotomy or autopsy at 48 hours after copulation, no certain cases of delayed ovulation were observed. There was, however, considerable histological evidence of partial activation, and there were many instances of activation combined with atresia of the ovum. Whether this atypical or activation-atresia represents the liberation of more, or less, LH than that required for simple partial maturation, cannot be determined from the present data, which are controversial on that point. The atypically atretic follicles appear to be similar to the cystic follicles produced in the rabbit by electrical stimulation too weak to induce ovulation (Ball and Hartman, 1939). Complete blocking of the release of LH was the exception rather than the rule even in the Dibenamine series, and no cases were observed in the atropine-treated animals. That this inability to block completely the ovulatory mechanism was attributable to the speed of transmission of the copulation stimulus in the rabbit is attested by the recent findings of Everett, Sawyer and Markee (unpublished) in the rat; in that animal the drugs may be administered before the onset of the neurogenic stimulus and complete blockages of the release of LH with either atropine or Dibenamine are common.

A mechanism involving cholinergic and adrenergic components, apparently in sequence, recalls conditions existing in the adrenal medulla and in sympathetic ganglia. In the adrenal medulla Feldberg, Minz and Tzudzimura (1934) demonstrated that adrenaline is released in response to acetylcholine liberated by the splanchnic nerve. We do not believe, however, that systemic adrenaline from the adrenal medulla normally stimulates the release of the hypophyseal LH, for two reasons: (1) No one has been able to induce ovulation in rabbits by the intravascular administration of adrenaline (Markee, Sawyer and Hollinshead, 1948); (2) cutting the pituitary stalk blocks copulation-induced ovulation in the rabbit without removing the major arterial supply (Brooks, 1938), which would import the systemic adrenaline.

In sympathetic ganglia (Dale, 1939) there is considerable evidence that a cholinergic mechanism (liberation of acetylcholine from the preganglionic endings) stimulates an adrenergic mechanism (transmission along the postganglionic fibers and liberation of adrenaline or sympathin from their endings). The cholinergic activity at the ganglion, as also in the adrenal medulla, is a nicotinic action of

acetylcholine, but still probably would be blocked by atropine in dosages as high as 30 mg./kg. (Abdon, 1940). However, it is not probable that the cervical sympathetics are included in the natural route of the coital stimulus, for several workers have removed them without interfering with copulation-induced ovulation (Vogt, 1931, 1933; Hinsey and Markee, 1933b). It is true that Friedgood and Pincus (1935) induced ovulation in the rabbit by stimulating the cervical sympathetic chain electrically, but they employed abnormally strong and prolonged ($\frac{1}{2}$ to $2\frac{1}{2}$ hrs.) stimulation. With stimuli of physiological intensities in the unanaesthetized rabbit, Markee, Sawyer and Hollinshead (1946) were unable to induce ovulation even when the periods of stimulation were prolonged.

Stimulation of the release of LH from the rabbit hypophysis by the injection of adrenaline directly into the gland (Markee, Sawyer and Hollinshead, 1948) has implicated an adrenergic mediator as the final link in neurogenic stimulation. If not from the adrenal medulla or cervical sympathetic nerves, what are its source and pathway? The finding by Westman and Jacobsohn (1937) and Brooks (1938) that rabbits with sectioned pituitary stalks would mate but not ovulate suggested to them that nerve fibers of the stalk transport the neurogenic stimuli which control the hypophysis. Westman and Jacobsohn (1937, 1938) demonstrated a similar importance of the stalk to normal gonadotrophic function in rats, but later Westman, Jacobsohn and Hillarp (1943) eliminated the essentiality of the abundant nerve fibers of the infundibular stalk. They reported numerous non-sympathetic nerve fibers entering the pars distalis through the tuberalis in the rat and also in the rabbit (Hillarp and Jacobsohn, 1943), and that as long as a tuberalis-distalis connection remained intact, gonadotrophic function remained undisturbed. Figure 20A interprets our results in terms of Westman, Jacobsohn and Hillarp's neural pathway with a central cholinergic synapse and a peripheral adrenergic nerve fiber. The central, hypothalamic site for the cholinergic component has a precedent in the recent work of Pickford (1947), who stimulated the release of posterior lobe hormone in the dog by injecting acetylcholine directly into the hypothalamus. Against the nerve fiber localization of the adrenergic component, however, are the following objections: (1) Nerve endings are very scarce in the pars distalis, regardless of source (Rasmussen, 1938, Green and Harris, 1947); (2) Green (1948) has described as reticulum, fibers similar to those diagnosed as nerves by Hillarp and Jacobsohn; (3) Markee, Sawyer and Hollinshead (1946) found the pars distalis practically inexcitable electrically, a condition that is not in keeping with a nerve-ending hypothesis. The finding has been confirmed by Harris (1948) who also reported that the infundibular stalk was electrically inexcitable. Both found that hypothalamic centers, which control the hypophysis, were sensitive to weak electrical stimuli

which were ineffective when applied directly to the adenohypophysis.

The arguments above as well as the findings of Westman, Jacobsohn and Hillarp, that the integrity of the tuberalis-distalis connection is essential for normal gonadotrophic control, may all be used as indirect evidence that the hypophyseal portal system is an important link in the neural control of the anterior pituitary. Wislocki and

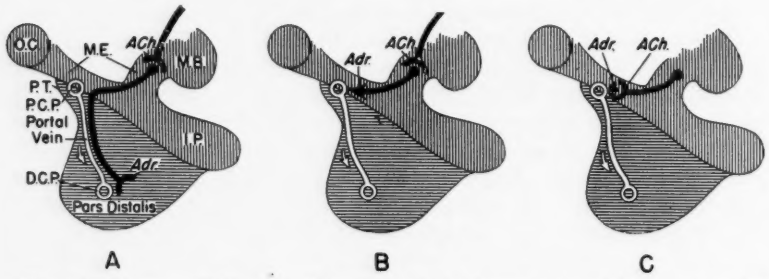

FIG. 20. Diagrammatic sagittal sections of the hypophysis with three possible schemes of the relationships of cholinergic and adrenergic components in the neurogenic stimulus for the release of LH. A. The adrenergic mediator is liberated in the pars distalis from nerve fibers entering via the pars tuberalis and accompanying the portal vessels; the cholinergic mechanism is located at hypothalamic synapses. The cells of the adenohypophysis would thus receive the adrenergic mediator directly from nerve endings. B. The adrenergic mediator is liberated from nerve endings in the median eminence directly into the proximal capillary plexus of the portal system; the cholinergic component has the same locus as in A. C. The adrenergic mediator is liberated into the proximal capillary plexus of the portal system from cells located in the median eminence; the cholinergic mediator is liberated in the median eminence from nerve fibers arising in the hypothalamus. In both B and C the cells of the pars distalis would receive the adrenergic mediator from the hypophyseal portal system at its distal capillary plexus. *ACh.*, source of acetylcholine. *Adr.*, source of adrenergic mediator. *D.C.P.*, distal capillary plexus. I. P., infundibular process. M.B., mamillary body. M.E., median eminence. O.C. optic chiasma. P.C.P., proximal capillary plexus. P.T., pars tuberalis.

King (1936) were the first to suggest that this system carries blood through the tuberal region in the direction of the pars distalis. Their findings have been confirmed and extended by Green and Harris (1947) and by Green (1948). Harris (1948) has recently summarized much of the evidence in favor of a neurovascular control of the adenohypophysis. Harris' suggestion that the portal system has rapid powers of regeneration may well account for the lack of impairment of gonadotrophic control following stalk section in the guinea pig (Dempsey, 1939) and in the rat (Dempsey and Uotila, 1940). Brolin (1947) has recently emphasized the importance of the tuberalis-distalis connection in the control of thyrotrophic hormone.

Figure 20, B and C, give two possible interpretations of the loci of the cholinergic and adrenergic components of the neural control with the portal system as the final pathway in each. Figure 20, B, has the cholinergic component at a central synapse in the hypothalamus and

the adrenergic agent liberated from nerve endings related to the proximal capillary plexus in the median eminence. An anatomical basis for the latter in the rabbit is found in Green and Harris' (1947) description of nerve endings in the walls of the looped and tufted vessels of the proximal capillary plexus.

Figure 20, C, places both cholinergic and adrenergic mechanisms peripherally, with acetylcholine from nerve endings stimulating the release of the adrenergic mediator from elements in the median eminence. The term "peripherally" is used advisedly for the median eminence is no longer considered part of the hypothalamus but of the posterior lobe of the hypophysis (Rioch, Wislocki and O'Leary, 1940). There is as yet no morphological basis for the location of the adrenergic component in such an hypothesis, but our work provides indirect physiological evidence for it. The fact that atropine is effective at all, when injected intravenously post coitum, suggests a peripheral locus of action for the cholinergic mediator. One might expect central neural synapses to be activated within a matter of milliseconds, whereas the injection of atropine and circulation time consume many seconds. The differences between effective atropine and Dibenamine injection times, again of the order of several seconds, implies that stimulation of the liberation of an effective amount of adrenergic mediator is also a slow process. Such time relationships would fit the liberation of adrenaline from cells around the proximal capillary plexus as well, at least, as from nerve endings. The observation of O'Connor (1947) and Green (1948), that the median eminence contains many pituicytes yet produces very little posterior lobe hormone, may have significance in this connection. Whether produced by nerve endings or cells, adrenaline is sufficiently stable to withstand inactivation during a short vascular journey, which is a characteristic probably not shared by acetylcholine in the absence of anticholinesterases.

SUMMARY AND CONCLUSIONS

Earlier work has demonstrated that an adrenergic mediator is involved in the neurogenic control of the release of luteinizing hormone from the rabbit hypophysis. Ovulation, which signifies the release of luteinizing hormone, was induced by instilling adrenaline, but not acetylcholine, directly into the anterior hypophysis, and copulation-induced ovulation was blocked by the rapid post-coital injection of the adrenolytic drug Dibenamine.

Like Dibenamine, atropine sulfate blocks ovulation when injected very quickly after copulation. However, to be equally effective atropine must be given earlier than Dibenamine: when injection was started at 15 seconds after the termination of copulation most of the atropine-treated rabbits ovulated, whereas most of the Dibenamine-injected animals failed to ovulate. Thus, a cholinergic (atropine-blocked) component of the copulation-initiated neurogenic stimulus

appears to precede the well established adrenergic component.

The ovarian follicles whose rupture has been blocked by atropine or Dibenamine almost invariably give histological evidence of partial activation. This includes characteristic preovulatory changes and also an atypical atresia of large follicles, in which the ova precede the granulosa in degeneration. After a follicle has ruptured, the normal development and function of the corpus luteum does not appear to be affected by atropine or Dibenamine.

The natural neurogenic stimulus at copulation traverses the pituitary stalk, and the final pathway, distal to the median eminence, is probably the hypophyseal portal system. Possibly in this system, as in the adrenal medulla and sympathetic ganglia, the cholinergic component stimulates the secretion of the adrenergic mediator, which in turn stimulates the hypophyseal cells to release luteinizing hormone.

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EFFECTS OF TESTOSTERONE PROPIONATE UPON SEXUAL LIBIDO AND THE PRODUCTION OF SEMEN AND SPERM IN THE RABBIT[1]

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INTRODUCTION

IT has been demonstrated that an exogenous supply of male hormone causes retardation and injury to the testes of the prepuberal rat but no marked involutionary changes in the adult rat (Moore and Price, 1932, 1937 and 1938). The injurious effect gradually declines with age and also tends to decline with increased dosage. A species difference in the action of androgen has been shown. The injection of male hormone stimulated spermatogenesis in the immature ground squirrel, and also in the mature ground squirrel during the season of testicular inactivity (Wells, 1936; Wells and Moore, 1936). An increase in the size of the testes (Lahr and Riddle, 1944) and a stimulation of spermatogenesis (Burger, 1945; Pfeiffer, 1947) was caused in the bird. In the opossum, however, androgenic treatments failed to show any consistent effect on the testicle between birth and maturity (Moore and Morgan, 1942).

A possible explanation for the injurious effect of the male hormone on rat testes was offered by Moore and Price (1932); they postulated that excessive male hormone causes the pituitary gland to release an insufficient amount of gonadotrophic hormone for testicular development and maintenance. The role of the pituitary gland in the development and regulation of the testes has been demonstrated by studies in which hypophysectomy caused an almost immediate cessation of spermatogenesis (Smith and Engle, 1927), and implantation of pituitary substance caused reactivation of testicular function in the hypophysectomized animal (Smith, 1930). The suppressing effect of male hormone upon gonadotrophic capacity of the pituitary gland has been reported by many investigators (McCullagh and Walsh, 1935; Moore and Price, 1937; Hertz and Meyer, 1937; Cutuly and Cutuly, 1938; Hamilton and Wolfe, 1938; Hellbaum and Greep, 1943).

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Male hormone alone will maintain or initiate spermatogenesis in the hypophysectomized rat (Walsh *et al.*, 1934; Nelson and Gallagher, 1936; Cutuly *et al.*, 1937; Cutuly and Cutuly, 1940; Nelson, 1941; Cutuly, 1942) and rabbit (Greep, 1939).

The lack of agreement concerning the effect of male hormone on testicular activity prompted the present study. Most previous studies were based upon histological characteristics of the reproductive organs. The present investigation is a study of the effect of male hormone at different dosages, upon various reproductive functions of the male rabbit. The changes in testicular activities are reflected by the changes in libido, and in production of seminal material and sperm, and in some of the physiological characteristics of the sperm.

MATERIALS AND METHODS

First Experiment: Twelve adult male rabbits were divided into 3 groups of 4 animals each according to the dosage of testosterone propionate, "TP," they were to receive. A "switch-back" experiment was designed. Two sequences of treatment were used in each of the 3 groups: in "sequence I," 2 animals of each group received TP in the first period of the experiment and no-TP in the second period; in "sequence II," the other 2 animals of the same group received no-TP in the first period and then were treated with hormone in the second period.

During their "treatment" periods, animals of the first group received 1 mg. of TP in 0.5 cc. of oil daily for 32 days (1 mg. group); the second group, 3 mg. daily also in 0.5 cc. of oil for 32 days (3 mg. group); and the last group 10 mg. TP daily in 1 cc. of oil for 20 days (10 mg. group). During the "no-treatment" periods, animals of the 1 mg. and 3 mg. groups received injections of 0.5 cc. of corn oil daily and those of the 10 mg. group 1 cc. daily. All injections, whether oil or oil and hormone, were made subcutaneously.

All animals were trained with an artificial vagina and "dummy" female. Collections of semen were made for a period of time at regular 4-day intervals previous to the experiment. In each period of the experiment, 7 "single-collection tests" were made at 4-day intervals for both the 1 mg. and 3 mg. groups and on the 8th collection day, an "endurance test" was made. This consisted of attempts at collection being made hourly for 10 hours. With the 10 mg. group, endurance tests were made regularly at 4-day intervals throughout each period of the experiment but in this case attempts at collection were made hourly for 6 hours.

The above 3 groups of animals were subjected to the experimental studies at different times during the Summer of 1947, the 1 mg. group was started July 16, the 3 mg. group July 29, and the 10 mg. group on August 21. No temperature control could be exercised in the animal quarters and the maximum atmospheric temperature during these experiments varied from 54°F. to 101°F. Because of the heat, single trial collections were made at 8:00 to 9:00 A.M. and the endurance tests were begun approximately at 5:00 A.M.

Second Experiment: During February and March 1948 the effects of different dosages of TP upon the reproductive performance of the male rabbit were reinvestigated. The temperature of the animal quarters varied from approximately 50°F. to 75°F. Some changes were made both in experimental

design and procedures. In this experiment, 8 adult males were divided into 4 groups of 2 animals each, designated as: control group, 0.5 mg. group, 3 mg. group and 20 mg. group.

The whole experiment consisted of 3 periods of 10 days each: (1) pre-treatment period, during which no hormone was administered to any group; (2) treatment period, during which blank injections were given daily to the control group and subcutaneous injections were given the experimental groups of 0.5 mg., 3 mg. and 20 mg. of testosterone propionate in oil; and (3) post-treatment period, during which no hormone or blank injections were given. Each animal was subjected to an endurance test every day which consisted of 6 trials with 30 minute intervals between trials.

RESULTS

The effect of TP upon libido: Libido or sexual desire of the animal, in the present studies, is indicated by the reaction time per trial and also by the number of attempts at ejaculation obtained in a given number of trials.

The reaction time was measured as the interval from contact of the animal with the artificial vagina until completion of the attempt at ejaculation. A maximum reaction time of 300 seconds in the first experiment and 180 seconds in the second experiment was permitted.

(1) *Reaction time*

In the first experiment, the average reaction time per trial for all dosage groups, whether in single-collection or endurance tests, was less when TP was being administered than when no hormone was given (Table 1-A). All the differences between TP-averages of reaction time and no-TP averages are statistically significant.

In the second experiment, the effects of treatment upon reaction time appeared very much the same as in the first experiment so far as the 0.5 mg. and 3 mg. groups were concerned. This was not the case, however, with the 20 mg. group (Table 1-B).

A decrease of reaction time occurred with the control group during the period in which the experimental animals received hormone (perhaps a continued training effect). The 0.5 mg. and 3 mg. groups, however, showed a significantly greater decrease of reaction time than the control group in the same period but the 20 mg. group showed less decline.

In the first experiment the average reaction time was greatly increased when the hormone treatment was terminated (Table 1-A: Sequence I). This was not true in the second experiment. In fact the experimental groups showed a further decrease in reaction time during the post-treatment period while the control group showed a slight increase. This discrepancy of results between the first and second experiment is not understood. It is assumed, however, that differences in the season and in the intensity of use of the males and differences in the males themselves are factors which will be involved in the eventual explanation.

(2) *Number of attempts at ejaculation*

It was found in both experiments, that animals receiving hormone injections averaged a larger number of attempts at ejaculation than animals receiving no hormone (Tables 1-A and 1-B). In the second experiment, the control group showed an increase in the number of

TABLE 1. THE EFFECT OF TP UPON LIBIDO

A. First experiment

Group	Sequence of treatment	Pd. I	Pd. II	Av. reaction time per trial (sec.)				Number and nature of attempts in endurance test			
				Single-col. test		Endurance test		Av. no. attempts		% attempts with no measurable amt. of semen	
				Pd. I	Pd. II	Pd. I	Pd. II	Pd. I	Pd. II	Pd. I	Pd. II
1 mg.	I	TP	No-TP	7.0	17.0	11.1	19.0	10	10	0	60
	II	No-TP	TP	34.0	15.8	199.4	132.7	5	7.5	60	40
	No-TP av. : TP-av.			25.5 : 11.4		109.2 : 71.9		7.5 : 8.8		60 : 20	
3 mg.	I	TP	No-TP	15.0	64.4	14.4	154.9	10	7	0	21
	II	No-TP	TP	21.1	8.1	61.6	13.3	9	10	11	0
	No-TP av. : TP-av.			42.7 : 11.5		108.2 : 13.8		8.0 : 10.0		16 : 0	
10 mg.	I	TP	No-TP			50.0	60.4	5.6	5.8	0	9
	II	No-TP	TP			132.8	68.2	4.3	5.8	5	2
	No-TP av. : TP-av.					96.6 : 59.1		5.0 : 5.7		7 : 1	

B. Second experiment

Group	Av. reaction time per trial (sec.)			Av. number of attempts per endurance test			% of attempts with no measurable amt. of semen		
	Pre-tr. period	Treat. period	Post-tr. period	Pre-tr. period	Treat. period	Post-tr. period	Pre-tr. period	Treat. period	Post-tr. period
Control	67	47	57	5.3	5.7	5.1	13	10	27
0.5 mg.	64	28	16	5.1	6.0	5.9	0	1	2
3 mg.	104	30	25	4.1	5.8	6.0	1	6	8
20 mg.	98	89	64	4.1	4.7	5.3	0	0	3

attempts during the period in which the experimental animals received hormone. This, again, is perhaps due to the continued training effect. However, the experimental groups showed a significantly greater increase in the number of attempts than did the control group in the same period.

A dosage difference in the number of attempts was noted in the post-treatment periods. In the first experiment, the 1 mg. group showed no change in the number of attempts from treatment to no-treatment period, the 3 mg. group showed a decrease, and the 10 mg. group showed a slight increase. In the second experiment, the control group showed a marked decrease in the number of attempts, while the 0.5 mg. group showed virtually no change, the 3 mg. group showed a slight increase, and the 20 mg. group showed a greater increase. The further increase of the number of attempts during the post-treatment period seems to have a positive correlation with the amount of hormone administered in the previous treatment period.

A further analysis of the first experiment reveals that the animals

while receiving TP gave relatively fewer attempts with no measurable amount of semen than when receiving no hormone whether the comparison is made between treatment and no-treatment on different animals within period, or on the same animal between different periods (Table 1-A). No such evidence was found in the second experiment, however, inasmuch as virtually all attempts of the experimental animals were successful in the pre-treatment period and this left no room for improvement during treatment (Table 1-B).

The effect of TP upon production of seminal material: The seminal material of the rabbit consists largely of a fluid portion, the "semen"; but occasionally additional material in the form of a transparent jelly mass, the "glairy" substance, also appears. The effects of TP upon the production of seminal material, therefore, are indicated by the volume of both semen and glairy substance.

(1) *Semen production*

In the first experiment, a difference of semen production was noticed between single-collection and endurance tests during the treatment period (Table 2-A). In the single-collection test, the TP-averages of semen volume were less than the no-TP averages in both 1 mg. and 3 mg. group. The latter difference is barely significant whereas the former is not. In the endurance tests, however, the TP-averages of semen volume were significantly greater than no-TP

TABLE 2. THE EFFECT OF TP UPON PRODUCTION OF SEMINAL MATERIAL

A. First experiment

Group	Sequence of treatment	Pd. I	Pd. II	Av. vol. semen per trial (cc.)				Av. vol. glairy substance per trial (cc.)			
				Single-col. test		Endurance test		Single-col. test		Endurance test	
				Pd. I	Pd. II	Pd. I	Pd. II	Pd. I	Pd. II	Pd. I	Pd. II
1 mg.	I	TP	No-TP	.51	.41	.26	.06	.55	0	.12	.01
	II	No-TP	TP	.45	.30	.06	.14	0	.02	0	.01
	No-TP av. : TP-av.			.43 : .40		.06 : .20		0 : .28		.01 : .06	
3 mg.	I	TP	No-TP	.52	.50	.29	.11	.42	.01	.35	0
	II	No-TP	TP	.69	.46	.20	.29	.16	1.07	.01	.18
	No-TP av. : TP-av.			.59 : .49		.15 : .29		.08	.75	.01 : .26	
10 mg.	I	TP	No-TP			.25	.14			.30	.04
	II	No-TP	TP			.16	.19			.01	.34
	No-TP av. : TP-av.					.15 : .22				.02 : .32	

B. Second experiment

Group	Av. vol. semen per trial (cc.)			Av. vol. glairy substance per trial (cc.)		
	Pre-treat. period	Treatment period	Post-treat. period	Pre-treat. period	Treatment period	Post-treat. period
Control	.14	.10	.09	.14	.13	.06
0.5 mg.	.25	.28	.22	.42	.48	.33
3 mg.	.22	.24	.19	.32	.50	.25
20 mg.	.39	.38	.43	.20	.41	.31

averages in all 3 groups. The discrepancy between the results of this experiment will be discussed in connection with the quantity of glairy substance. The increase of semen volume as shown in the endurance tests during the hormone treatment period appeared to be greatest in the 1 mg. group, less in the 3 mg. group, and least in the 10 mg. group. The apparent negative correlation between dosage of hormone and semen volume in this experiment is unexplained.

In the second experiment, the 0.5 mg. and 3 mg. group also tended to show an increase in the volume of semen during the treatment period; there was no noticeable change in the 20 mg. group; and the control group showed a decrease (Table 2-B). These differences, however, were not statistically significant.

Changes in semen production from treatment to post-treatment period were observed. In the first experiment, a decrease of semen volume was noted both in the single-collection and in the endurance tests when the hormone treatment was terminated (Table 2-A). The 1 mg. group showed the greatest decrease of semen volume, the 3 mg. group next, and the 10 mg. least. In the second experiment, a decrease of semen volume during the post-treatment period was observed only in the 0.5 and 3 mg. groups, while the 20 mg. group showed an increase, and the control group showed no change (Table 2-B). The changes of semen production from treatment to post-treatment were significantly different between groups.

(2) *Glairy substance production*

In the first experiment, the volume of glairy substance produced by the animals prior to treatment was much less than that produced by the animals in the pre-treatment period of the second experiment (Table 2-A: "No-TP averages" in Sequence II and Table 2-B: averages in pre-treatment period). This difference may have been due to season or to the individuality of the animals.

The hormone administration caused a significant increase in production of glairy substance in both experiments with the exception of the 1 mg. group in the endurance test of the first experiment (Tables 2-A: Sequence II and II-B). A positive correlation was found between the dosage of hormone and the production of glairy substance. In the single-collection tests of the first experiment, the 1 mg. group gave less glairy substance than the 3 mg. group; in the endurance tests, the 10 mg. group produced the greatest volume of glairy substance, the 3 mg. group next, and the 1 mg. group least. In the second experiment, the same positive relationship was found in that the 20 mg. group produced the greatest volume of glairy substance, the 3 mg. group next, and the 0.5 mg. group least, while the control group showed no noticeable change. The differences between groups were highly significant.

The discontinuation of the exogenous hormone supply caused an

immediate drop in glairy substance in the first experiment (Table 2-A). In the second experiment, a drop was noted in both control and experimental groups; however, the 0.5 mg. and 3 mg. group showed a less decrease than the control group, and the 20 mg. group showed a still lesser decrease than either the 0.5 mg. or the 3 mg. group (Table 2-B).

A much greater volume of glairy substance was produced in the first ejaculation than in the later ones of the endurance test. This may explain the inconsistent results of semen production between the single-collection and the endurance tests of the 1 mg. and 3 mg. groups of the first experiment. A negative relationship was found to exist between the volume of glairy substance and the volume of semen in the first ejaculation. It was as though the large volume of glairy substance in the first ejaculation interfered with the complete ejaculation of the seminal fluid; while in the second ejaculation, the small amount of glairy substance caused little or no interference with complete ejaculation, and consequently, a greater volume of semen was produced.

The effect of TP upon the sperm production

(1) *Sperm concentration*

In the first experiment, a marked decrease in sperm concentration occurred during the second period in all groups with the exception of "Sequence I" of the 10 mg. group (Table 3-A). The general "period" differences were highly significant.

TABLE 3. THE EFFECT OF TP UPON SPERM PRODUCTION

A. First experiment

Group	Sequence of treatment	Pd. I	Pd. II	Av. sperm conc. (1000/mm³)				Av. no. sperm per trial (millions)			
				Single-col. test		Endurance test		Single-col. test		Endurance test	
				Pd. I	Pd. II	Pd. I	Pd. II	Pd. I	Pd. II	Pd. I	Pd. II
1 mg.	I	TP	No-TP	487.9	7.2	47.9	0.2	263.9	3.2	18.5	0.1
	II	No-TP	TP	456.9	120.4	292.0	8.8	178.4	38.2	30.7	2.3
	No-TP av. : TP av.			232.0 : 304.1		146.1 : 28.4		151.0 : 90.8		15.5 : 10.4	
3 mg.	I	TP	No-TP	317.9	78.6	257.1	61.3	169.6	39.3	84.5	6.5
	II	No-TP	TP	384.0	333.1	278.9	10.7	230.4	115.1	56.3	37.0
	No-TP av. : TP av.			231.3 : 322.5		170.1 : 133.9		134.9 : 142.3		31.4 : 61.2	
10 mg.	I	TP	No-TP			229.8	258.8			58.0	34.0
	II	No-TP	TP			429.9	365.2			66.3	68.8
	No-TP av. : TP av.					344.4 : 297.5				50.1 : 63.4	

B. Second experiment

Group	Av. sperm conc. (1000/mm³)			Av. no. sperm per trial (millions)		
	Pre-treat. period	Treatment period	Post-treat. period	Pre-treat. period	Treatment period	Post-treat. period
Control	134	167	221	18	17	18
0.5 mg.	101	70	109	22	20	24
3 mg.	161	83	132	31	20	21
20 mg.	68	69	57	20	23	22

In the single-collection tests of the first experiment, the TP-averages of sperm concentration were greater than no-TP averages in both 1 mg. and 3 mg. groups; the difference for the latter group was highly significant while that for the former was not. However, this was not the case in the endurance test. The TP-averages of sperm concentration, in all 3 groups, were slightly less than the no-TP averages, but these differences were not significant.

In the second experiment, the 0.5 mg. and 3 mg. groups showed a decrease in the sperm concentration, the 20 mg. group showed no change, while the control group showed an increase (III-B). The differences between groups were highly significant.

Changes of sperm concentration during the post-treatment period seemed to differ between the two experiments. In the first experiment, a decrease in sperm concentration was found in the 1 mg. and 3 mg. groups, in both single-collection and endurance tests, while the 10 mg. group showed a slight increase. In the second experiment, an increase of sperm concentration was found in the control, 0.5 mg. and 3 mg. groups, while the 20 mg. group showed a decrease.

(2) *Number of spermatozoa*

Analyses of the results showed great "period" differences in the first experiment. In fact, variation due to this cause in the 1 mg. and 3 mg. groups was greater than that associated with any other one factor. High summer temperatures prevailed during the first period with these groups and the effects of this hot weather appeared one to two weeks after its onset rather than at the beginning, so far as sperm number is concerned.

There was a marked decrease in the number of spermatozoa during the second period of the experiment regardless of whether the animals were or were not receiving hormone treatment. This was true in all groups with the exception of the "Sequence II" animals of the 10 mg. group (Table 3-A). In the single-collection tests of this experiment, the TP-average of the number of spermatozoa was significantly less than the no-TP average in the 1 mg. group, but no significant change was shown in the 3 mg. group. In the endurance tests, the TP-average of the number of spermatozoa was also less than the no-TP average in the 1 mg. group, but the TP-average was higher than no-TP average in the 3 mg. group. The latter difference was highly significant while the former was not. The 10 mg. group showed no significant difference in the number of spermatozoa between TP- and no-TP averages.

In the second experiment also, no definite changes in the number of spermatozoa were shown, and no significant differences were found between groups (Table 3-B). Thus the general situation is that testosterone propionate did not modify the total sperm production. Various factors, however, interacted in a compensatory manner to bring this about. Concentration of sperm sometimes declined but

increased volume compensated for it. The increase of volume was obtained because of the endurance test; more ejaculates were obtained from the treated animals than from the untreated and this built up the semen volume to furnish approximately the same total number of sperm.

The effect of TP upon motility of spermatozoa in different dilutions. In these tests, the semen was first diluted with modified Krebs solution (Lardy and Phillips, 1942) into 8 successive dilutions from 1:100 to 1:12,800. The semen suspensions were kept in a water bath at 38° C. for 30 minutes, and then the percentages of the progressively motile spermatozoa were estimated. This test was done only in the single-collection tests of the first experiment, i.e., the 1 mg. and 3 mg. groups.

It was found that the TP-averages of motility of spermatozoa in 8 dilutions was higher than no-TP averages in both groups (Table 4). The differences were highly significant.

TABLE 4. THE EFFECT OF TP UPON MOTILITY OF SPERMATOZOA IN 8 SUCCESSIVE DILUTIONS FROM 1:100 TO 1:12,800

Group	Sequence of treatment	Pd. I	Pd. II	Av. % mot. in 8 successive dilutions		Av. no. of dilutions showing mot.	
				Pd. I	Pd. II	Pd. I	Pd. II
1 mg.	I	TP	No-TP	49.1	14.0	7.9	4.6
	II	No-TP	TP	40.1	39.4	6.9	6.1
	No-TP av. : TP av.			27.0	: 44.2	5.7	: 7.0
3 mg.	I	TP	No-TP	36.6	22.6	6.9	5.0
	II	No-TP	TP	38.7	43.1	6.5	7.3
	No-TP av. : TP av.			30.6	: 39.8	5.7	: 7.1

The resistance of the spermatozoa, as indicated by the average number of successive dilutions in which the spermatoza showed progressive motion, appeared to be higher during the treatment period than when no hormone was being given (Table 4). The significance of the difference was not determined in the 1 mg. group because of incomplete data; however, the difference in the 3 mg. group was highly significant.

SUMMARY

The effect of testosterone propionate upon reproductive performance of adult male rabbits was studied in two experiments involving 20 animals. The first experiment was run in July to September 1947 and the second experiment in February and March 1948.

Sexual activity was increased rather consistently in both experiments. This was indicated by a decrease in the average interval to

obtain an attempt at ejaculation (reaction time) and also by an increase in the number of attempts at ejaculation.

The quantity of the "glairy" fraction of the semen was definitely increased in both experiments but no consistent effect of treatment was noted upon the volume of the fluid portion of the semen.

No definite evidence was obtained for an effect of treatment upon the sperm concentration in the fluid portion of the semen. In some instances there appeared to be a slight increase and in others a slight decrease.

The total number of spermatozoa produced during the periods of treatment, likewise, was not definitely affected. Inasmuch as there was a larger number of ejaculates during treatment, it would appear that the number per ejaculate was decreased slightly and the total number was maintained in part by virtue of more ejaculates having been given.

The average motility of the sperm when tested in different dilutions with Kreb's solution was studied in the first experiment only. Treatment appeared to improve average motility in successive dilutions as high as 1 to 12,800.

Post-treatment effects were not consistent between the two experiments. A greater continuation of the effects of treatment occurred in the second experiment when endurance tests were being run daily rather than at 4-day intervals as in the first experiment and when the weather was cold rather than hot as in the first case. Both of these factors, frequency of use and environmental temperatures, need further study.

Testosterone propionate was used effectively in increasing sexual libido of male rabbits without marked deleterious effects upon sperm production.

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THE RESPONSE OF THE THYROID GLAND IN NORMAL HUMAN SUBJECTS TO THE ADMINISTRATION OF THYROTROPIN, AS SHOWN BY STUDIES WITH I^{131}*

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THE actions of thyrotropin in various laboratory animals have been studied in considerable detail. Following its administration the thyroid gland becomes hypertrophied and hyperplastic, the content of protein-bound iodine decreases, but there is an increase in the inorganic iodine fraction. The circulating protein-bound iodine and the basal metabolic rate are elevated. An increased capacity of the gland to fix iodine has been demonstrated by the use of I^{131}.

Since none of these methods has been generally applicable to the study of normal human beings, and since relatively pure preparations of the hormone have not been readily available, little information has accumulated regarding the actions of thyrotropin in man.

In the present study radioiodine methods originally designed for demonstrating the action of thyroid inhibiting substances were adapted to study the influence of single doses of thyrotropin on the several phases of iodine metabolism in the normal human thyroid gland.

METHODS

The methods used were those which have previously been described (Stanley and Astwood 1947, 1948). The total iodine accumulation was studied by administering to each subject by mouth a tracer dose of I^{131}, diluted in normal saline solution, without added carrier. The collection of radioiodine by the thyroid gland was detected by serial counting at thirty- to sixty-minute intervals with a shielded Geiger-Müller counter in contact with the skin over the thyroid gland above the sternal notch. The resulting data, expressed as counts per second per 100 microcuries of administered I^{131}, were plotted versus the square root of the time in minutes, thus converting the parabolic uptake curve to a straight line. The slope of this straight line, referred to as the accumulation gradient, served as an index of the rate of iodine uptake. Measurements during the first one to two hours of the study

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allowed a fairly accurate prediction of the course of uptake for the remainder of the period; deviations from this course could be related to the effects of administered inhibiting or stimulating substances (Figure 1).

This approximate straight-line relationship was found to hold for eight hours or more in normal subjects, but the uptake was virtually completed

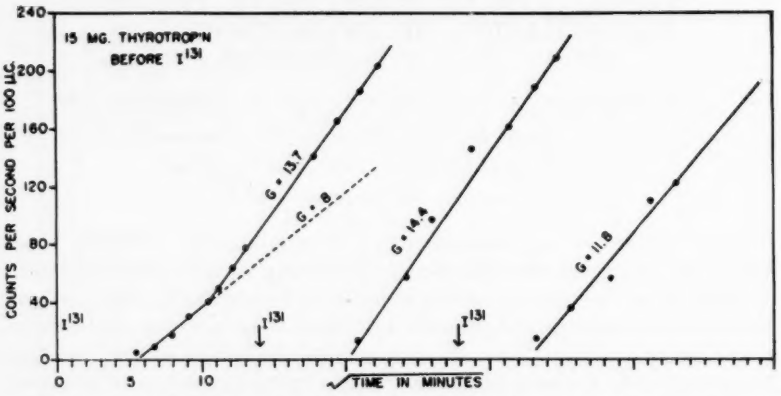

FIG. 1. The influence of thyrotropin on the accumulation of radioiodine in a normal young woman. Fifteen milligrams of thyrotropin was administered intravenously five hours and forty-one minutes before the I^{131} was taken by mouth. During the two hours following the tracer dose the course of uptake was established with an accumulation gradient of 8. At two hours and six minutes after the I^{131}, or seven hours and forty-seven minutes after thyrotropin was given, the rate of accumulation increased to a gradient of 13.7. On the second day the uptake following a second tracer was higher (accumulation gradient—14.4), but was decreasing on the third day.

by the end of twenty-four hours in these and in the group given thyrotropin. Very little change occurred for several days thereafter. Consequently, it was possible to make a second tracer study as early as twenty-four hours after the first.

The uptake of iodide ion was measured after the administration of mercaptoimidazole, an antithyroid drug of the thiouracil type, in quantities sufficient to inhibit as completely as possible the organic binding of iodine in the thyroid. Usually 100 milligrams of mercaptoimidazole were given one to two hours before the I^{131} was given, followed by 50 milligrams every eight hours for the duration of the experiment. It could be readily demonstrated that the radioactive iodine entrapped was *iodide* and that organic binding did not occur to an appreciable extent except in the instances mentioned below. The administration of 1 to 2 grams of potassium thiocyanate caused a rapid discharge of the thyroid iodide and permitted identification of that portion of the total iodine which was in ionic form (Figure 2). In plotting these results, an ordinary linear time scale was adequate for the abscissa. In some instances the logarithm of the thyroid iodide content was plotted against time. Since the iodide in the thyroid was in a state of equilibrium with the blood iodide, they both decreased in a logarithmic fashion as the blood level was reduced by renal excretion.

In order to estimate the iodide-concentrating capacity, it was necessary

to measure quantitatively the radioactivity in the thyroid gland as accurately as possible. The best approximation that could be obtained was calculated from determinations made at a relatively large distance (26.5 cm.) from the thyroid using a sensitive gamma counter. In view of the fact that it was not possible to shield completely the radiation from other body tissues,

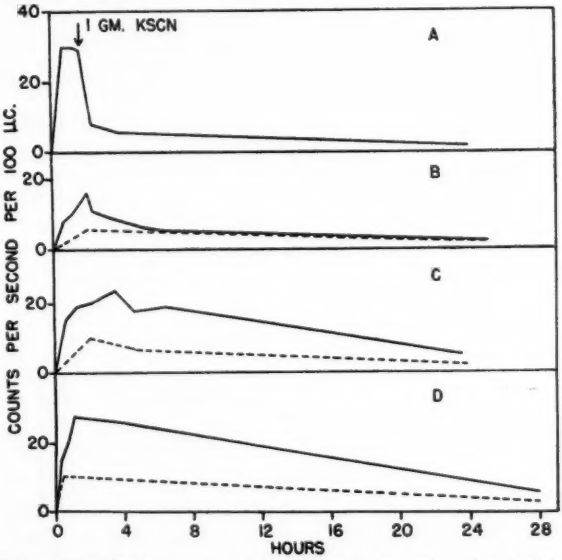

FIG. 2. The accumulation and subsequent fate of radioactive iodide ion in the thyroid glands of four normal persons is shown by solid lines. For comparison, the radioactivity over the left anterior chest are indicated by dotted lines. In all cases the administration of an effective dose of mercaptoimidazole preceded the dose of tracer by about an hour. Proof that the tracer was in the form of the iodide ion was provided by the rapid discharge from the gland of virtually all the contained radioactivity following administration of 1 gram of potassium thiocyanate in subject A. In those individuals in whom complete inhibition of organic binding was maintained, and who were not given thiocyanate, there was a gradual loss of radioiodide from the gland during twenty-four to forty-eight hours. Compare with Fig. 4.

and because the factor of scattering could not be accurately estimated, the determination of the total radioactive iodine in the thyroid gland was possibly in error by 20 to 25 per cent. In order to calculate the quantity of radioactive iodide in 1 gram of thyroid tissue, the weight of the gland was assumed to be 25 grams in each subject. This estimate might also have been in error by 50 to 100 per cent. However, it was thought that any errors which occurred were constant in the same subject, so that the comparative determinations were quite accurate.

In some experiments additional tracer doses were given at intervals of one or two days. The rates of accumulation of these additional tracers were calculated in such a manner that the residual radioactivity from former doses was subtracted from the subsequent readings. Thus, the plotted figures show only those increments of the total radioactivity in the gland produced

by the dose of I^{131} administered for the respective test. In most such multiple-dose experiments each dose of I^{131} was 20 to 50 microcuries, depending upon the rate of uptake expected, but all values were expressed in terms of 100 microcuries.

The thyrotropin used[2] was freshly dissolved in 1 to 3 milliliters of distilled water or normal saline and administered as a single dose intramuscularly, or intravenously.

Two groups of subjects of both sexes were given thyrotropin during the study. The first was composed of seventeen individuals who had no abnormalities of thyroid or pituitary function, and who had been taking no drugs known to influence these glands. Patients who were taking thyroid in varying amounts made up the second group of six people. On the basis of history these latter patients were considered to have had normal thyroid function before they were given desiccated thyroid; this opinion was substantiated by the course of events following the discontinuation of the medication. Numerous additional subjects of both types received no thyrotropin and served as controls.

RESULTS

Total Iodine Accumulation in Normal Subjects: For purposes of comparison, the findings in 265 normal subjects tested for various purposes during the past two years were summarized. The majority of subjects were between fifteen and fifty years of age, and most were females. The accumulation gradients varied between 2 and 13 in 96.5 per cent of instances. The ten subjects whose gradients fell outside these limits had higher values. Eight of these unusually high values were observed during the early work with radioiodine; there is reason to believe that due to a difference in methods of standardization the size of the tracer dose was about 50 per cent higher than it should have been.

Since the data to be presented were derived in many instances from studies with multiple doses of tracer radioiodine, the constancy of the accumulation gradient in repeated tests on untreated control patients was investigated. Thirty-six subjects were studied more than once. When several months elapsed between the tests, rather wide variations were encountered in some instances. Consequently an additional group of four normal subjects was tested on successive or alternate days. As shown in Table 1, the rates of uptake on repeated tests were quite uniform. The close agreement in these results indicated that this method could be used to study changes in thyroid activity over the course of several days.

Effects of Thyroid Medication: The normal subjects who were receiving 4 to 8 grains of thyroid daily exhibited a complete or very

[2] The thyrotropin was generously supplied through the kindness of Dr. John R. Mote of the Armour Laboratories, Chicago, Illinois. It was a purified preparation from beef anterior pituitary glands with a stated potency of 10 to 20 Waleszek-Koch chick units per milligram. This unit is defined as the total amount which, when injected twice daily for three days, will reduce the thyroid iodine of the chick by 50 per cent.

nearly complete suppression of iodine uptake (Figure 3). The suppressive effect of chronic treatment with smaller doses of desiccated thyroid has not been studied quantitatively, but from the few tests which were made it was evident that there was considerable individual variation. In one subject $1\frac{1}{2}$ grains daily produced nearly complete inhibition which continued for several weeks after treatment was stopped. While 3 grains daily in one individual was sufficient to produce only a moderate inhibition, in another given this dose the suppression was complete.

TABLE 1. THE ACCUMULATION GRADIENTS CALCULATED ON SUCCESSIVE OR ALTERNATE DAYS IN FOUR NORMAL SUBJECTS

Days	Gradient				
	1	2	3	4	5
Subject A	4.2	4.2	3.7		
Subject B	9.3		9.0		9.1
Subject C	6.3		6.4		6.2
Subject D	10.1	10.0	10.2		

Influence of Thyrotropin: The effect of thyrotropin on the *total iodine accumulation* by the thyroid was studied in fifteen patients who were considered to have normal or potentially normal thyroid function before suppression by desiccated thyroid.

Examples of the type of response which was observed are shown in Figures 1 and 3. Two individuals received single doses of I^{131} in conjunction with the thyrotropin, while the others were given repeated tracer doses. After a tracer dose of radioiodine, the course of uptake by the thyroid region followed a distinctive pattern during the first eight to twelve hours, and changes in the rate of uptake were easily detected during this interval. When 15 or 30 milligrams of thyrotropin were given by intravenous or intramuscular injection during the course of the accumulation of a tracer dose, no effect was observed during the ensuing six or seven hours (Figure 3, C). Subsequent tests were, therefore, made by administering the thyrotropin before the tracer study was begun. Two subjects studied in this way exhibited an abrupt increase in the accumulation gradients at about eight hours after the injection of thyrotropin (Figure 1). In others the increase was more gradual; it was just detectable at eight hours (Figure 3, A), and a more rapid acceleration ensued thirteen to fourteen hours following the injection (Figure 3, A and B).

During the second twenty-four-hour period following the injection of 15 milligrams, stimulation of iodine accumulation was at its height. During the third day, there was a decline toward the preinjection value. The subjects who continued taking thyroid and whose initial accumulation gradients approached zero exhibited very low rates of uptake after the fourth day. This indicated that the stimulat-

ing effect of the hormone had completely subsided by the end of the fourth day following injection (Figure 3, A). After the administration of 30 milligrams, the stimulating action was still demonstrable for five days (Figure 3 C).

In one patient (Figure 3, B), whose dose of 3 grains daily of desic-

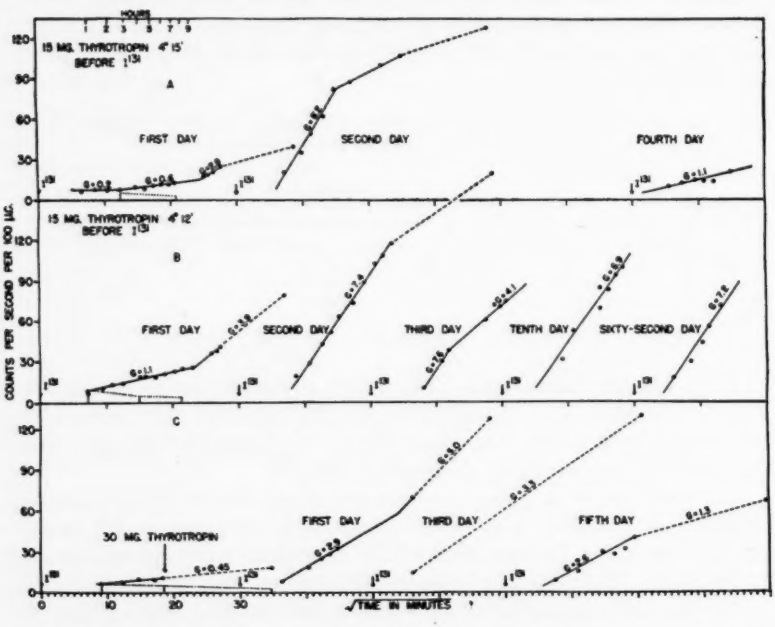

FIG. 3. The influence of thyrotropin on the iodine uptake in three normal persons who had been taking thyroid. Patient A had taken 5 grains daily, B, 3 grains daily, and C, 6 grains daily. The medication of subjects A and C was continued throughout the study, but in B the thyroid was discontinued ten days before the experiment. Each patient received a single dose of thyrotropin at the time indicated. In patient A an increased rate of uptake began eight hours after injection of the thyrotropin. This became more pronounced at fourteen hours and was maximal on the second day. On the fourth day the effect had nearly completely subsided. The sequence of changes observed in subject C was similar, but the effects of the thyrotropin were still evident on the fifth day. In B, the uptake continued essentially unchanged after the third day, presumably under the influence of endogenous thyrotropin.

cated thyroid had been discontinued ten days prior to the first test, a different sequence was observed. The control uptake was low, but not zero, indicating that function was not completely suppressed. The maximal augmentation of uptake occurred on the second day following the injection of 15 milligrams of thyrotropin, and the increased rate of accumulation was only slightly diminished on the third day. One week later, with no treatment of any sort in the meanwhile, the iodine uptake was again at the high level of the second day. On the sixty-second day after the initial injection thyroid function was still

normal without further treatment. Presumably a normal thyroid-pituitary relationship was re-established at about the time the exogenous thyrotropin was administered.

The Iodide Uptake and the Thyroid-to-Blood-Iodide Ratio were studied in eight normal subjects who had not been given thyroid. Inhibition of organic binding of iodine in the thyroid gland was continuously maintained with mercaptoimidazole throughout each series of tests. The influence of thyrotropin on the thyroid iodide content was observed over the course of twenty-four hours or longer.

When organic binding of iodine is prevented, a tracer dose of I^{131} labels the iodide ion in the thyroid and in the body as a whole. Equilibrium is quickly established so that one to three hours after the tracer is given the radioiodide in the thyroid region is at a maximum. Thereafter the thyroid radioiodide gradually declines as the body's I^{131} is slowly lost in the urine and replaced by I^{127} from endogenous and exogenous sources (Figures 2 and 5 A, B).

When thyrotropin was given, the rate of decline in the thyroid's radiodine was markedly modified; there was either much less of a decrease, no decline, or as much as a 90 per cent increase in the iodide levels one day later (Figure 4). To gain further information about the magnitude of the increased capability of the thyroid for concentrating iodide, the amounts of radioactivity in the serum were correlated with the thyroid radioiodide determined at intervals before and following the action of thyrotropin. When no thyrotropin was given, it was observed that the ratio of the iodide in the thyroid to the iodide in the serum remained nearly constant for one to two days (Figure 5, A and B). In the instance of subject A shown in Figure 5, the concentration of iodine in the thyroid during the second day was somewhat higher than expected, and it is possible that this was caused by the organic binding of a small amount of iodine. After the administration of thyrotropin, the ratio was constant for approximately eight hours and then increased markedly. In one experiment the change was progressive until the thirty-first hour, after which it remained approximately constant for fifteen hours (Figure 5, C). The capacity of the thyroid for concentrating iodide was increased to 6.5 times the control value in this patient.

Other subjects were given multiple tracer doses to study the influence of single injections of thyrotropin on the iodide uptake. In one patient (Figure 4, C) the course during the first $23\frac{1}{2}$ hours after 30 milligrams of thyrotropin was as described above; between $7\frac{1}{2}$ hours and $23\frac{1}{2}$ hours there was a rise in radioactivity in the gland even though the radioactivity in the body as a whole had fallen markedly. The greatly increased iodide-concentrating capacity was then studied by a second tracer dose which revealed a maximal concentration eight times that of the first day. This high concentration diminished unusually quickly during the balance of the second twenty-four-hour pe-

riod at the termination of which the radioactive iodide level from the second tracer was less than it was at the end of the first twenty-four-hour period. At the beginning of the third day the maximal accumulation from a third tracer dose, forty-eight hours after the thyrotropin, was slightly less than three times the control value.

In a second subject (Figure 4, B) studied for 42½ hours after a single tracer dose, the usual gradual decline in radioactive iodide did

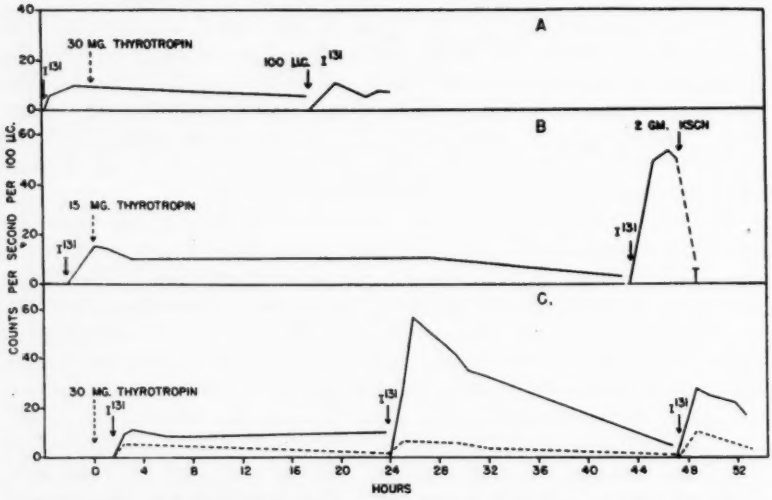

FIG. 4. Tracer studies on the iodide content of the thyroid in the three normal subjects above were performed in the same way as those shown in Figure 2, except for the injection of thyrotropin. During the twenty-four hours following the administration of thyrotropin the expected decrease in radioactivity in the thyroid was partially prevented, and in B and C there was actually a slight increase during this period. Twenty-four to forty-eight hours after the injection of thyrotropin there was an increased capacity for the concentration of iodide. In B, the nearly complete loss of radioactivity after thiocyanate showed that the increased accumulation was actually iodide ion and not organically bound iodine.

not occur until thirty hours after the thyrotropin was given. A second tracer revealed that the iodide content of the thyroid gland was 3.4 times higher than it was before the thyrotropin effect began. At the end of the test the prompt discharge of 94 per cent of the radioactivity from the gland after 2 grams of potassium thiocyanate indicated that virtually all of the iodine was still in the form of iodide, and that only a negligible degree of organic binding had occurred.

Effect of Thyrotropin on the Discharge of Stored Thyroid Hormone: Following injections of thyrotropin in the chick, Keating *et al.* showed a rapid loss of stored hormone from the thyroid; this occurred during the first twenty-four hours, while a significant increase in iodine ac-

cumulation did not take place until the second day. This suggested that an emptying of the follicles of their contents was a prerequisite for an increased iodine uptake.

It was of interest to determine whether a single injection of thyrotropin would result in a measurable loss of stored hormone. In the subjects who were given 15 or 30 milligrams of thyrotropin, the maximal accumulations attained during the first twenty-four hours after the tracer were not followed by significant declines during the following two to four days. Two other subjects were first given a tracer dose of I^{131} and then, when the uptake had ceased, were given

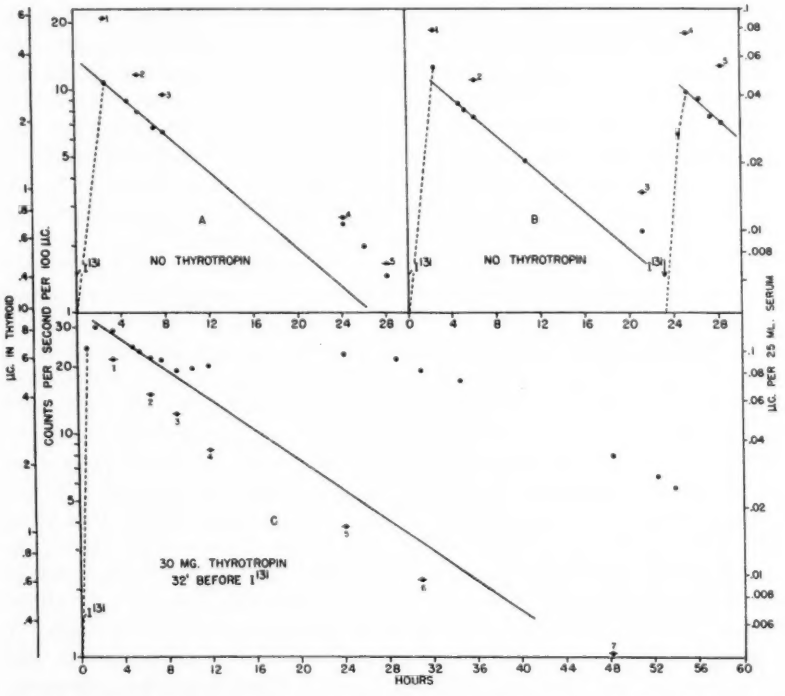

FIG. 5. The influence of thyrotropin on the capacity of the normal thyroid gland to concentrate iodide ion. Each of the three normal subjects above received repeated doses of mercaptoimidazole to inhibit the organic binding of iodine. The solid circles show the radioactivity of the thyroid region, and the crossed circles indicate the radioactivity in the serum. The actual thyroid:serum iodide ratios were:

A. (1) 34, (2) 43, (3) 43.2, (4) *59.9*, (5) *78.1*

B. (1) 43.9, (2) 43.3, (3) 42.8, (4) 34.6, (5) 35.2

C. (1) 85.5, (2) 92.5, (3) 99.5, (4) 153, (5) 378, (6) 554, (7) 494, (8) 543.

It will be seen in (A) that the last two ratios are higher than the first three. This is due to a higher than expected thyroid concentration, probably caused by the organic binding of a small amount of iodine. In subject C under the influence of thyrotropin there was a marked increase in the ratio beginning at about eight hours after injection and attaining a value of 6.47 times the control values at thirty-one hours.

a single dose of 30 milligrams of thyrotropin. Serial determinations extending over thirteen hours and seven days, respectively, revealed no significant loss of the stored I^{131}.

In view of the large store of hormone contained in the normal human thyroid, it was perhaps to be expected that no loss of the labeled organic iodine would be detectable following such a brief period of stimulation.

DISCUSSION

The latent period of eight or more hours between the time of thyrotropin administration and the first detectable increase in thyroid activity is difficult to understand. Numerous experiments with various anterior pituitary hormones have shown that frequent injections are necessary to achieve maximal effects. Most anterior pituitary hormones are far more effective if the daily dose is subdivided and given every few hours, suggesting that hormones of this type are rapidly eliminated from the circulation. It would seem likely that the maximal stimulus to the thyroid gland following a single injection of thyrotropin would occur during the first hour or so and that by eight hours, when the first detectable increase in function is noted, the quantity of circulating thyrotropin would be already markedly decreased. Certainly it would be reasonable to suppose that by the second or third day, when evidences of stimulation are still clearly manifest, the injected thyrotropin has largely been eliminated. It would seem as if thyrotropin in its action upon the thyroid cell merely initiates a sequence of changes which then proceeds without its further influence.

It was of interest to find that the two aspects of thyroid function studied were influenced by thyrotropin in a similar manner. The increased organic binding of iodine was first detectable eight hours after the thyrotropin was given and became maximal some hours later. As far as could be determined, the increased capacity of the thyroid to concentrate iodide ion exhibited identical time relationships. As these two aspects of thyroid activity were stimulated simultaneously, it would not be reasonable to assume that one was primary and the other secondary. Rather it would appear that these two processes merely reflected an increased activity on the part of the thyroid cell induced by the injected thyrotropin. When the capacity of the cell to bind iodine was increased, an increased quantity of iodide ion was at the same time provided; when the cell was caused to concentrate more iodide, it at the same time could convert it more rapidly into an organic form. Inasmuch as the increase in iodide-concentrating capacity occurred when organic binding was inhibited, the binding process was obviously unessential for the phenomenon of augmentation of iodide concentration. It could not, however, be stated that the increase in iodide concentration did not facilitate a more rapid binding

of this iodine, but various considerations suggested that this would not be a large factor.

It would be of interest to know whether the increased rate of secretion of thyroid hormone follows the same temporal pattern after thyrotropin as the other two aspects of thyroid activity which have been studied here. From studies of radioautographs Leblond and Gross have concluded that all of the functions of the thyroid cell proceed simultaneously. They have pointed out that, in analogy to the intestinal mucosa, the thyroid epithelium could easily secrete a protein hormone into the follicle at the same time as it digested and secreted the stored hormone into the blood. If this concept is correct, it would suggest that thyrotropin stimulates the several components of thyroid function simultaneously; it enhances the capacity of the thyroid cell to concentrate iodide ion, to bind iodine to protein, to secrete thyroid protein into the follicle, to digest stored hormone, and to secrete it into the blood stream.

From the previous studies on the iodide-concentrating capacity of the thyroid gland (Stanley and Astwood, 1948), it was suggested that an increased ability of the thyroid to concentrate iodide ion correlated in some way with hypertrophy and hyperplasia. The experiments reported in the present communication suggested that an increased capacity for iodide was a reflection of the action of thyrotropin on thyroid tissue. The ratio of the concentration of iodide ion in thyroid tissue to that in the serum was found to vary between 34 and 94, and averaged 50, in three normal persons in the present study. The means by which this estimate was made was subject to a considerable margin of error, but it was of interest that the ratio was of the same magnitude as that found by VanderLaan and VanderLaan (1947) in normal rats. Here a more exact measurement was possible and the ratio averaged 25. Likewise, the high value of 554 shown by one patient given thyrotropin might be compared with the mean of 240 found in rats whose glands were made hyperplastic by the administration of propylthiouracil.

The studies reported above have practical implications. One is able to determine whether or not a patient being treated with thyroid does or does not have a potentially functional thyroid gland. It is common clinical experience that when euthyroid patients are treated with thyroid, the withdrawal of medication is sometimes followed by transient symptoms and signs of hypothyroidism. This phenomenon has been carefully studied by Riggs, Man, and Winkler, who showed that when large doses of thyroid were discontinued the metabolic rate, serum cholesterol level, and the concentration of serum-precipitable iodine changed from values in the range of mild hyperthyroidism to those of myxedema. It was only after four to six weeks that these criteria of thyroid function reverted to normal. The use of thyrotropin in conjunction with radioactive iodine makes it possible to

determine quickly whether the thyroid gland of the thyroid-treated patient is present and normally responsive.

SUMMARY

Studies with I^{131} of the effects of a single injection of 15 or 30 milligrams of thyrotropin in 23 normal subjects have been made. For eight hours after the injection no effect on the rate of iodine uptake by the thyroid could be detected, but thereafter a marked acceleration, becoming maximum in twenty-four to forty-eight hours, was observed. The rate of turnover of iodine returned to the pre-injection level in four to five days. This sequence of events was still more clearly exhibited by individuals whose thyroids had been compensatorily suppressed by thyroid medication. When the organic binding of iodine was prevented by the use of mercaptoimdazole, it could be shown that the iodide-concentrating capacity of the thyroid was similarly increased by thyrotropin and the time relationships of this increase were the same as those of the total iodine accumulation. Following a single injection of thyrotropin, it was not possible to detect any loss of stored thyroid hormone.

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THE TRANSPORT OF PROGESTERONE IN BLOOD[1]

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THIS study was undertaken with the objective of determining the order of concentration of progesterone in the blood of certain representative mammalian species and of ascertaining the form in which this hormone is transported in the blood. It seems generally supposed that steroid hormones in the blood are intimately associated with protein (Edsall, 1947), although evidence for this relationship has, so far as we are aware, been presented only for estrogen (Häussler, 1936; Mühlbock, 1939; Rakoff, Paschkis, and Cantarow, 1943; Szego and Roberts, 1946, 1947; Roberts and Szego, 1946; Boettiger, 1946).

METHODS

No effort was made to examine any reproductive state; the only consideration was to be reasonably confident that the blood to be examined would contain progesterone. Accordingly, blood was obtained from pseudopregnant rabbits (virgins, six or seven days after injection of gonadotrophin), a rhesus monkey in the luteal phase of the menstrual cycle, a woman considered to be in the eighth week of pregnancy, and a group of mice five days after mating. Examination revealed that not more than one-half of the donor mice were pregnant. The blood from the woman and the monkey was drawn from a superficial vein; that from the rabbits and mice was obtained by cardiac puncture. Sodium citrate was employed as the anticoagulant.

The assays of progesterone were done by the intrauterine injection method in mice (Hooker and Forbes, 1947), which detects 0.0002 μg. of progesterone in a standard volume of 0.0006 ml. of vehicle, a concentration of 0.33 μg. per ml. The sensitivity of the test is the same whether sesame oil or 0.9 per cent saline is the vehicle (Forbes and Hooker, 1948). The concentration of progesterone was ascertained by injecting serial dilutions of the specimen to be tested, the greatest dilution giving a positive test being considered to reflect the concentration. For example, if a plasma sample gave a positive test when diluted 18 times and a negative test when diluted 20 times, the concentration is recorded as 6.0 μg. per ml. (18×0.33). It will be apparent that the precision of the value depended upon the gradations in the dilutions tested.

The values for raw whole blood and for raw plasma were obtained by testing serial dilutions of each made with 0.9 per cent saline. Separation of

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the free and bound progesterone in plasma was attempted in two ways. In the first the sample was extracted with ether, a procedure used by Rakoff, Paschkis, and Cantarow (1943) to obtain the free estrogen in blood. A measured, small amount of sesame oil was added to the ethereal extract, and the ether was then removed by distillation under diminished pressure, leaving the material extracted by the ether in solution in the oil. This fraction was labelled "free" progesterone. Following extraction with ether the plasma residue was submitted to hydrolysis by bringing it to pH 0.5–1.0 with concentrated hydrochloric acid and immersing in boiling water for six hours. After cooling, the mixture was extracted with ether and the dissolved material was incorporated in a measured amount of sesame oil as above. This fraction was labelled "bound" progesterone. In three instances the plasma after extraction with ether was tested for progesterone before hydrolysis.

The second method of separation was a modification of that used by Szego and Roberts (1947) for separating free and protein-bound estrogen. Here the plasma was added slowly with stirring to approximately ten volumes of acetone at 3°C. to precipitate the proteins. After standing overnight, the supernatant was pipetted off, and the precipitate was washed three times at the centrifuge with a 1:1 mixture of acetone and ether. An effort was made to keep the material cold. The washings were combined with the supernatant, a measured amount of sesame oil was added, and the ether and acetone were removed by distillation under diminished pressure. Most of the water of the plasma of course remained, and the oil was allowed to separate by standing or by centrifugation. This fraction was labelled "free" progesterone. The proteins were allowed to dry in air, powdered, and suspended or dissolved in distilled water. The suspension or solution was acidified with concentrated HCl to pH 0.5 to 1.0 and immersed in boiling water for six hours. After cooling, the mixture was extracted with ether, and the dissolved material was incorporated in sesame oil as described above. This fraction was labelled "bound" progesterone.

In certain instances raw plasma and raw whole blood were submitted to the same hydrolytic procedure without first removing the free progesterone. The objective was to determine whether the procedure destroys progesterone. In two instances the proteins in a sample of plasma were precipitated by tungstic acid, and the filtrate was assayed. In one instance a mass of plasma protein precipitated by acetone was suspended in water and tested without hydrolysis.

RESULTS

The results are summarized in Table 1. The columns are arranged from left to right in the chronological order in which the samples were assayed; the increase in agreement reflects improvement in technical proficiency and greater foresight in making the dilutions for test. The failure to get exact values for the mouse blood was the result of not testing intermediate dilutions.

The values found for the concentration of progesterone in raw plasma ranged from 5.3 to 8.0 μg. per ml. The values for whole blood are less exact, but the apparent range was from 4.0 to 6.0 μg. per ml. The progesterone revealed by these tests appears to have been en-

tirely in the plasma. On the basis of the values for whole blood and the hematocrit for the second rabbit, the woman, and the monkey, plasma concentrations of 6.0, 5.4, and 6.5 μg. per ml., respectively, would be expected if all of the progesterone revealed were in the plasma. The

TABLE 1. BLOOD PROGESTERONE LEVELS, μG/CC.

Species, status	Rabbit, pseudopregnant				Mouse preg. ±	Man preg. 8 weeks	Monkey luteal phase
	7th day	7th day	7th day	6th day			
Hematocrit	26	20	27		27	35.7	28
Raw whole blood	>2.6 <5.3	5.0			5–10	4.0	5.0
Raw plasma	5.3	6.0		8.0	5–10	5.3	6.0
Protein free filtrate, plasma	3.7	5.0					
Ether extract, plasma	4.3	5.3			5–10	5.3	6.0
Acetone supernatant, plasma			6.6		5–10	5.5	6.0
Ether insol., plasma	0	0					0
Acetone ppt., plasma			0				
Hydrolysate, ether insol.	0.3	0.5			0.5	0.2	0.2
Hydrolysate, acetone ppt.			0.9		0.8	0.2	0.2
Hydrolysate, whole blood				6.0	7.3–9.3	4.3	5.3
Hydrolysate, raw plasma		6.6–8.2			8.3–9.3	5.3	6.0
Bound, per cent of total	5.3	7.7	12.0			3.5	3.2

observed values of 6.0, 5.3, and 6.0 μg. per ml., respectively, are in satisfactory agreement with the expected values.

The values for the ethereal and acetone extracts are in good agreement with each other and with the values for the same raw plasmas. Apparently, therefore, the progesterone in plasma that manifested itself upon intrauterine injection was quantitatively extracted by both solvents. Moreover, neither solvent removed progesterone that was inactive while in the plasma.

Although tested only twice, and both times early in the study, the amount of active progesterone remaining in the filtrate of plasma after precipitation of the proteins was comparable to that in the raw plasma and that extracted by ether.

Tests of three plasma samples after removal of the ether-soluble material revealed no progesterone in concentrations as high as 0.33 μg. per ml. (the minimal concentration that the assay detects), and are in consequence recorded as being negative. Similarly, a suspension of acetone-precipitated plasma protein from the third rabbit exhibited no activity. When, however, the plasma after extraction with ether was submitted to acid hydrolysis and concentrated somewhat in oil solution, progesterone in amounts ranging from 0.2 to 0.5 μg. per ml. of original plasma was revealed. Similarly, acid hydrolysis of the plasma proteins precipitated by acetone revealed progesterone in amounts ranging from 0.2 to 0.9 μg. per ml. of original plasma.

If the progesterone extracted by ether and acetone is considered to be free progesterone and that revealing itself only after hydrolysis

of the proteins is considered to be bound, the latter fraction constituted 3.2 to 12.0 per cent of the total progesterone. The average was 6.5 per cent.

The low values for bound progesterone suggested the possibility that the procedure employed for hydrolysis of the proteins might inactivate bound progesterone almost as rapidly as it was freed. The quantity of progesterone found after hydrolysis of raw whole blood and raw plasma, however, was in every instance no less than the values for free progesterone in raw whole blood and in raw plasma. In some instances the values were almost exactly the sum of the free and bound fractions. Hence it seems unlikely that the hydrolytic procedures destroyed progesterone.

DISCUSSION

The similarity of the levels of progesterone from species to species may represent nothing more than chance selection of physiological states with comparable levels of progesterone. The data suggest, however, that during active luteal function the blood levels of progesterone may be comparable in several species.

A plasma level of progesterone varying around 6.0 μg. per ml. is somewhat higher than the available estimates for other steroid hormones. McCullagh and Osborne (1938) found approximately 4 I.U. of androgenic activity in 100 ml. of human plasma. If the actuve substance be assumed to be testosterone, the concentration was 0.6 μg. per ml. In whole blood from bulls, Womack and Koch (1932) found 1 I.U. of androgenic activity in 600 ml., a concentration of 0.025 μg. per ml. if the active substance were testosterone. In blood from pregnant women the highest concentration of estrogen, calculated as estrone, found by Szego and Roberts (1947) was 0.9 μg. per ml., and by Goldberger and Frank (1942) was 0.13 μg. per ml. The higher level of progesterone is consistent with its lower activity if it is permissible to compare quite different actions of different hormones.

The finding that approximately 10 per cent of the total progesterone is "bound" contrasts with the observation that approximately 65 per cent of the estrogen in blood is protein-bound (Rakoff, Paschkis, and Cantarow, 1943; Szego and Roberts, 1946). Denaturation of protein by the procedures employed in removing the free progesterone is an obvious possibility to account for the low value for bound progesterone. Precipitation by cold acetone and the maintenance of low temperatures throughout the separation of the free fraction should minimize denaturation, however; the free values obtained by this method were similar to those given by extraction with ether and to the activity of untreated raw plasma. On the other hand, it is possible that all of the progesterone is bound to protein, most of it so loosely that the methods employed would not distinguish it from free progesterone. Such a circumstance would also be unlike that reported for estrogen in blood. It will be apparent that the objective

here was to compare the transport of progesterone with that reported for estrogen, and the observations cannot elucidate the nature of the association with protein. Indeed, the bound fraction may have been conjugated, for example as sulfate or glucuronide, and not in any way related to protein, inasmuch as the methods employed probably would not differentiate protein-bound and conjugated progesterone. A possible, but not insistent argument against conjugation is that conjugated progesterone has not apparently been reported to occur. An oblique argument that the binding was to protein is that this fraction was revealed by procedures used to demonstrate protein-bound estrogen, and estrogen has been separated from purified proteins (Roberts and Szego, 1946).

Protein-bound estrogen in blood has been suggested to act as a sort of reservoir, with an equilibrium between the free and protein-bound moieties (Szego and Roberts, 1946). This suggestion seems unlikely for bound progesterone. The amount of this fraction appears to be too low, relatively, and it seems incapable of freeing itself in a period of 48 hours in a uterine segment. Indeed, binding is apparently a means for the hepatic inactivation of progesterone in the mouse (Forbes and Hooker, unpublished). It would be desirable to know whether bound progesterone is dialyzable as is estrogen (Szego and Roberts, 1946). The low concentration of this fraction, the low solubility of progesterone in water below body temperature (Forbes and Hooker, 1948), and the rapid disappearance of progesterone from plasma stored at body temperature (Hooker and Forbes, unpublished) would make the determination difficult.

In the blood of the four species examined progesterone is thus apparently transported entirely in the plasma and is primarily free rather than bound to protein. The concentrations of free progesterone, and indeed of total progesterone, in no instance exceeded the solubility of this substance in water at body temperature (Forbes and Hooker, 1948). It is apparent, therefore, that no special or complex mechanism is needed to dissolve the amounts of progesterone found in blood, and none was indicated. Various mechanisms are possible and may be operating (Masson and Selye (1945) report a much higher solubility of progesterone in serum in vitro), but their necessity is not evident. The same situation appears to obtain for estrogen. The highest concentration found by Szego and Roberts (1947), calculated as estrone, was 0.9 μg. per ml. of whole blood, which could not be greater than 1.35 μg. per ml. of plasma (even assuming a hematocrit of 50), whereas estrone dissolves in water to the extent of 2.1 μg. per ml. (Doisy, 1939). The binding of roughly two-thirds of the total estrogen to protein (Rakoff, Paschkis, and Cantarow, 1943; Szego and Roberts, 1946) may serve to increase the solubility of this substance, but binding may be unnecessary for this purpose and, indeed, have quite another function.

Obviously, three values for progesterone in blood may be deter-

mined—free, bound, and total. The first may be learned by assaying raw blood or plasma, the last by assaying hydrolyzed raw blood or plasma, while determination of the bound fraction necessitates fractionation and hydrolysis. The labor and time involved are greatest when the bound value is determined and least when only the free value is wanted. If the desired end is an assessment of the humoral environment of a target organ, the free value appears at the moment to be the only value of importance. The significance of the bound fraction is unknown, but it has thus far been quite low, and of indicated consequence only in the metabolism of progesterone.

SUMMARY

The blood levels of progesterone during one phase of luteal activity in rabbits, mice, a monkey, and a pregnant woman were determined by a bioassay method that detected a concentration of 0.33 μg. per ml. In the samples examined the total progesterone was between 4.0 and 8.0 μg. per ml. of whole blood, and was located entirely in the plasma.

Progesterone in the plasma apparently was present in two forms. Roughly 90 per cent was free, and the remainder was bound to protein or conjugated. The bound or conjugated fraction was inactive as tested, and was revealed only after partial hydrolysis of the plasma proteins. Fractionation was accomplished by methods that have been employed to separate free and protein-bound estrogen in blood.

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THE EFFECT OF GROWTH HORMONE ON THE INORGANIC PHOSPHORUS LEVELS IN THE PLASMA[1]

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THE fact that the hypophysis may play an important role in the control of inorganic phosphorus in the animal has been known for some time. Kobayashi (1931) and Schijo (1934) have shown that hypophysectomy in dogs causes a reduction of the inorganic phosphorus of the blood. Irradiation of the rabbit's pituitary also brought about a decrease in blood phosphorus (Cannavo and Beninato, 1934). In rats, the removal of the pituitary reduces markedly the serum inorganic phosphorus level (Anderson and Castler, 1938; Jones and Shinowara, 1942).

The earlier work of Teel and Watkins (1929) indicated that anterior pituitary extract produced an increase of inorganic phosphorus in the blood of dogs. These results were later extended by Gerschman and Marenzi (1935) who found that the blood inorganic phosphorus level changes from 4.25 mg. to 7.88 mg. per cent as the result of pituitary extract injections. The clinical data of Reifenstein *et al.* (1946) showed that the serum inorganic phosphorus level is elevated in both acromegalic patients and growing children. These experiments seem to indicate that growth hormone in the anterior pituitary extract is responsible for the changes of inorganic phosphorus content in the plasma.

Experimental. Male rats of the Long-Evans strain were used. The animals, fasted from 16 to 20 hours, were anesthetized with sodium amytal and blood was taken from the inferior vena cava with heparin solution as the anticoagulative agent. The method of Berenblum and Chain (1938) was employed for phosphorus determination. Growth hormone was prepared by the method previously described (Li, Evans and Simpson, 1945).

The change of plasma inorganic phosphorus level with age. The in-

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organic phosphorus concentration in the plasma of male rats[3] at different ages is summarized in Table 1. It is evident that young growing rats have more inorganic phosphorus in their blood in comparison with that found in later stages of life. The inorganic phosphorus in the plasma is maintained at the same level from 15 days to 55 days of

TABLE 1. INORGANIC PHOSPHORUS CONTENT IN PLASMA OF MALE RATS AT DIFFERENT AGES

Age	No. of rats	Body weight	Inorganic phosphorus
days		gm.	mg. per 100 cc. plasma
15	15	31.0± 0.92*	10.6±0.08
27	9	59.2± 3.0	10.9±0.45
40	5	139.4± 0.8	10.7±0.55
55	5	217.6±18.5	11.4±0.57
68	6	222.3± 8.3	8.2±0.34
73	6	253.5± 9.4	8.2±0.42
82	6	264.7±17.9	7.8±0.28
119	5	330.0±18.5	7.7±0.31

* Mean ± standard deviation.

age. When the animals grow older, the level decreases from 11.4 to 7.7 mg. per 100 cc. plasma and then becomes constant.

The effect of hypophysectomy. In confirmation of earlier investigators, hypophysectomy causes a lowering of inorganic phosphorus content in the plasma of rats. It may be seen in Table 2 that the phosphorus concentration changes from 10.7 mg. to 8.1 mg. per 100 cc. plasma after the pituitary has been removed for 7 days. The inorganic phosphorus level continues to fall but becomes constant at 2 weeks postoperative.

The influence of growth hormone. Male rats were hypophysectomized at the age of 40 days; intraperitoneal injections of growth hor-

TABLE 2. INORGANIC PHOSPHORUS CONTENT IN PLASMA OF HYPOPHYSECTOMIZED MALE RATS AT DIFFERENT POSTOPERATIVE PERIODS
40 days old at operation

Post-operative	No. of rats	Body weight		Inorganic phosphorus
		Initial	Final	
days		gm.	gm.	mg. per 100 cc. plasma
0	5	139.4±0.8*		10.7±0.55
7	4	140.0±6.8	118.2± 8.1	8.1±0.33
14	26	138.3±2.9	115.4± 3.4	6.6±0.22
28	7	141.2±3.0	118.6±10.0	5.6±0.20
42	3	145.0±6.2	115.7± 4.2	6.9±0.24

* Mean+standard deviation.

[3] The plasma inorganic phosphorus level of mature female rats was also determined and found somewhat lower than that occurring in male rats of similar age. From 17 female rats of ages from 91 to 111 days, an average value of 6.5 ±0.41 mg. inorganic phosphorus in 100 cc. plasma was obtained. As shown in Table 1, male rats 119 days old have 7.7 ±0.31 mg. per cent of plasma inorganic phosphorus.

TABLE 3. EFFECT OF GROWTH HORMONE ON INORGANIC PHOSPHORUS CONTENT IN PLASMA OF HYPOPHYSECTOMIZED MALE RATS*

Daily dose	No. of rats	Body weight			Inorganic phosphorus
		Initial	Final†	Gain	
mg.		gm.	gm.	gm.	mg. per 100 cc. plasma
0.00	26	138.3 ± 2.9‡	115.4 ± 3.4	−22.9	6.6 ± 0.22
0.05	5	144.6 ± 5.3	152.0 ± 1.5	7.4	8.2 ± 0.41
0.10	8	145.5 ± 4.1	150.4 ± 4.6	3.9	10.6 ± 0.37
0.20	8	134.8 ± 2.6	146.0 ± 2.6	11.2	10.3 ± 0.36

* Rats were operated at 40 days of age; intraperitoneal injections began on the day of operation and lasted for 15 days.
† Final body weights were obtained just before fasting.
‡ Mean ± standard deviation.

mone began on the day of operation and were continued for 15 days. Table 3 shows that a daily dose of 0.10 mg. is sufficient to prevent the fall of the inorganic phosphorus concentration in the plasma and to keep it at a normal level. It is of note that doubling of this daily dose does not elevate the phosphorus level above the initial level. However, 0.05 mg. of the hormone injected daily for 15 days will give a higher plasma inorganic phosphorus content than that in the controls.

TABLE 4. EFFECT OF CHRONIC INJECTION OF GROWTH HORMONE ON INORGANIC PHOSPHORUS CONTENT IN PLASMA OF HYPOPHYSECTOMIZED FEMALE RATS

Experiment	Body weight			Inorganic phosphorus
	Initial	Final	Gain	
	gm.	gm.	gm.	mg. per 100 cc. plasma
Injected*	68.6 ± 0.54†	409.6 ± 27.6	341.0	5.2 ± 0.41
Control	66.0 ± 0.50	103.6 ± 6.3	37.6	2.7 ± 0.20

* Rats 26 to 28 days of age at operation; 12 days postoperative at the beginning of injection; 0.1 mg. daily dose for the first 111 days, 0.2 mg. 270 days, and 0.4 mg. the last 22 days.
† Mean ± standard deviation.

In another experiment, hypophysectomized female rats were injected with growth hormone for 437 days; the plasma of these animals was analyzed for inorganic phosphorus. Results are summarized in Table 4. The control group gained 37.5 gm. having an inorganic phosphorus content of 2.7 mg. per 100 cc. plasma, while the injected animals gained 341.0 gm. and the plasma inorganic phosphorus level almost doubled that of the control.

Since young growing animals have a higher inorganic phosphorus concentration in the plasma and since hypophysectomy causes a stoppage of body growth with an accompanied lowering of the phosphorus level, the plasma inorganic phosphorus content may therefore be

taken as an indication of growth. The augmenting effect of growth hormone is hence to be anticipated. Until the actual significance of inorganic phosphorus on the process of growth is known, we offer no interpretation for the results herein reported.

SUMMARY

The inorganic phosphorus level in the rat plasma is found to decrease with age. Hypophysectomy causes a decrease of the plasma inorganic phosphorus content, while injections with growth hormone prevent this fall and even elevate the phosphorus level above that of the control.

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INFLUENCE OF GROWTH AND ADRENOCORTICOTROPIC HORMONES ON THE BODY COMPOSITION OF HYPOPHYSECTOMIZED RATS[1]

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A. THE INFLUENCE OF GROWTH HORMONE

THE changes in the body composition of animals after treatment with pituitary growth extracts have been studied by several investigators. Both Downs (1930) and Wadehn (1932) obtained accelerated growth in mice with a pituitary preparation and found that the treated animals contained more water and protein but less fat than did the controls. Bierring and Nielsen (1932) induced increase in body weight in rats by an alkaline anterior pituitary extract and showed that the body weight increment was not entirely due to retention of water. Using the pair-fed technique, Lee and Schaffer (1934) conclusively demonstrated that the growth in rats caused by injection of pituitary growth extract is chiefly due to increase in the protein and water content and that there is a decrease in fat. Recent studies of Young (1945) have confirmed these findings.

Since all preceding experiments have been carried out with only partially purified growth-promoting extracts, it is naturally difficult to decide as to whether such results were due to the growth hormone itself or to other contaminating proteins. In addition, earlier investigators did not employ hypophysectomized animals. The results obtained might conceivably have been caused by the influence of the injected extract upon the animals' pituitary. For these reasons, we have determined again the chief chemical constituents—water, lipids and protein of the carcass of hypophysectomized rats treated with the pure anterior hypophyseal growth hormone as isolated by Li *et al.* (1945).

EXPERIMENTAL

Female rats, 26 days old at hypophysectomy and 26 days postoperative, were restricted to a daily intake of approximately 3.0 gms. of food and injected with 0.10 mg. of the growth hormone daily in-

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traperitoneally for 30 days. Before the animals were autopsied, they were fasted for 24 hours. The entire gastrointestinal tract and internal organs were removed and discarded. The carcass was weighed and frozen in a cold room kept at −20°C. Moisture was determined by drying the frozen carcass in vacuum to constant weight. The dried carcass was then ground and the resulting uniform mixture was used for the determination of the total lipids and of total nitrogen. The fat was extracted thoroughly by means of petroleum ether in a Soxhlet apparatus. The ether extract was evaporated and the residue dried to constant weight. The weight of the dry residue was taken as the measure of the total fat in the tissue. The nitrogen content of the carcass was determined by the micro-Kjeldahl method, a factor of 6.25 being used for conversion into the protein value.

RESULTS

As shown in Table 1, the average gain in body weight of 7 treated animals was 42.5 gms., whereas that of the 7 controls was only 8.9 gms. The composition of the carcass is altered by the growth hormone treatment. Data in Table 1 indicates that the carcass of the treated

TABLE 1. COMPOSITION OF THE CARCASS OF HYPOPHYSECTOMIZED FEMALE RATS UNDER RESTRICTED FOOD INTAKE AFTER GROWTH HORMONE TREATMENT
7 animals in each experiment

Experiment	Body weight		Weight per 100 gm. carcass		
	Initial	Autopsy	Water	Fat	Protein
Injected[1]	61.6 ± 1.9[2]	104.1 ± 1.9	70.32 ± 0.68 (<0.001)[3]	4.44 ± 0.41 (<0.01)	19.24 ± 0.39 (<0.02)
Control	63.7 ± 2.2	72.6 ± 1.6	66.58 ± 0.84	9.39 ± 0.96	17.80 ± 0.14

[1] Hypophysectomized female rats, 26 to 28 days old at operation and 26 days post-operative, were restricted to approximately 3 gms. food intake daily. A daily dose of 0.1 mg. growth hormone was used for 30 days.
[2] Mean ± standard deviation.
[3] Fisher's p values.

animals contained more water and protein but less fat as compared with controls. For instance, the total lipids were reduced from 9.39 gms. to 4.44 gms. per 100 gms. tissue, while the protein content increased from 17.80 gms. to 19.24 gms. The retention of water is also marked; it changed from 66.58 to 70.32 per cent. These differences in the percentage composition of the tissues between injected and control rats were all highly significant as analyzed by the statistical method of Fisher (1938).

In another experiment, male rats hypophysectomized at 40 days of age were used. Intraperitoneal daily injections of 0.2 mg. growth hormone began on the day of operation and continued for 15 days. The animals were allowed to eat ad libitum but were fasted 24 hours before autopsy. It may be seen in Table 2 that the uninjected (control) animals lost 13.4 gms. in body weight while the animals receiving growth hormone gained 22.0 gms. The fat content in the carcass of

the treated animals was significantly lower than that in the controls: the value being 5.70 gms. versus 8.66 gms. per 100 gms. tissue. The percentage content of protein is somewhat higher in the treated carcass but not statistically significant. Furthermore, the amount of

TABLE 2. COMPOSITION OF THE CARCASS OF HYPOPHYSECTOMIZED MALE RATS AFTER INJECTIONS OF GROWTH HORMONE

Experiment	No. of rats	Body weight		Weight per 100 gm. carcass		
		Onset	Autopsy	Water	Fat	Protein
		gm.	gm.			
Growth Hormone[1]	8	126.0±1.0[2]	148.0±0.3	70.55±0.30 (<0.001)[3]	5.70±0.38 (<0.01)	18.70±0.27 (0.20)
Control	12	125.4±1.5	112.0±2.9	66.20±0.41	8.66±0.66	17.72±0.54

[1] Male rats, hypophysectomized at 40 days of age, were injected with 0.20 mg. growth hormone daily for 15 days immediately after operation; animals were fasted 24 hours before autopsy.
[2] Mean ± standard deviation.
[3] Fisher's p values.

water in the treated rats is highly increased. Thus, the results with male animals are essentially the same as those obtained with hypophysectomized female rats.

DISCUSSION

One of the characteristic functions of growth hormone is to retain nitrogen either in normal rats, in diabetic rats, or in rats with bilateral femoral fractures (Li, and Evans, 1947). It has further been shown that growth hormone causes a decrease of free amino acid in the plasma of rats (Li, unpublished data). It seems therefore reasonable to assume that the body growth induced by growth hormone treatment is at least partly due to the result of the acceleration of protein synthesis. The results just presented are in line with this assumption. The data show that *growth hormone causes a rise in the protein and water content with a lowering of the fat content of the body and* it is immaterial whether the animals are restricted in food intake or allowed to eat ad libitum. In fact, Young (1945) has shown that a restriction in food intake exaggerates the changes in body composition which normally follow pituitary treatment in the rat.

Since the rapid accumulation of protein is a distinctive pattern occurring in the process of growth (Moulton, 1923), there is no doubt that *the gain in body weight induced by growth hormone injections represents true growth.* It is well known that embryonic tissues containing high proportion of water and are relatively low in fat; it may be inferred that the tissue formed by the action of growth hormone is embryonic in nature.

Although it is clear that the total fat content of the body is reduced as the result of growth hormone injections, the nature of these changes remain unknown. The lowering of the fat content by growth hormone may reflect only a reduction in neutral fat, for it has been shown (Williams, Galbraith, Kaucher and Macy, 1945) that about 90 per cent of the total fat in rats consists of triglycerides. Since structural lipids, such as phospholipid, cholesterol and cerebrocides, are

essential components of cellular structure and since some of these lipids are known to increase with growth (Williams, Galbraith, Kaucher and Macy, 1945; Williams, Galbraith, Kaucher, Moyer Richards and Macy 1945), it may be anticipated that further studies will show that the concentration of "structural" lipids in tissues may actually be increased by growth hormone injections.

B. THE INFLUENCE OF ADRENOCORTICOTROPIC HORMONE

Evidence has accumulated indicating that adrenocorticotropic hormone may be regarded as a specific growth-inhibiting substance (Li and Evans, 1947). When it was injected simultaneously with growth hormone in hypophysectomized rats a counteraction exists between these two hormones (Marx, Simpson, Li and Evans, 1943). Since growth hormone has been shown to cause an increase in the protein content and a reduction in the fat in the body of hypophysectomized rats, it was of interest to similarly investigate the body composition of animals after adrenocorticotropic hormone treatment.

EXPERIMENTAL

Female rats, hypophysectomized on 26 days of age and 26 days postoperative, were injected intraperitoneally twice daily with a 1.0 mg. total daily dose of pure adrenocorticotropic hormone for 10 days. The animals were restricted to approximately 4.5 gm. of diet daily and fasted 24 hours before autopsy. The hormone was prepared by the method previously described (Li, Simpson and Evans, 1943). The carcass analysis was carried out in the same manner as outlined in the growth hormone experiment.

RESULTS

The body weight and its chemical composition of experimental and control rats are summarized in Table 3. As to be expected, there is no essential difference in body weight between the experimental and control groups. The changes in body composition are due to the loss of water and an increase of fat content from 8.62 to 11.57 gms. per 100 gms. carcass. The protein content is practically unchanged. It may be noted that the changes in fat and water content are statistically significant.

TABLE 3. COMPOSITION OF THE CARCASS OF HYPOPHYSECTOMIZED FEMALE RATS AFTER INJECTIONS OF ADRENOCORTICOTROPIC HORMONE (ACTH)

Experiment	No of rats	Body weight		Weight per 100 gm. carcass		
		Onset	Autopsy	Water	Fat	Protein
		gm.	gm.			
ACTH[1]	6	74.3±2.1[2]	71.3±1.7	64.55±0.52	11.57±0.61	16.89±0.32
				(<0.01)[3]	(<0.01)	
Control	8	79.6±2.1	76.5±1.5	67.96±0.44	8.62±0.39	16.73±0.43

[1] Female rats, hypophysectomized on 26 days of age and 26 days postoperative, were injected twice daily with 1.0 mg. total daily dose for 10 days; animals were under restricted food intake (approximately 4.5 gm. daily) and fasted 24 hours before autopsy.
[2] Mean ± standard deviation.
[3] Fisher's p values.

DISCUSSION

Since the secretory activity of the adrenal cortex enhances protein catabolism (Li and Evans, 1947; Ingle, 1944). it may not be unreasonable to expect that the body of adrenocorticotropic hormone treated animals would contain less protein. Experiments just reported show no change in protein content of the treated rats, although the animals definitely deposit more fat. It may be that the carcass contains actually less protein but our determinations did not differentiate protein from non-protein nitrogen.

The analytical values recorded in Table 3 clearly demonstrate that *adrenocorticotropic hormone induces a rise in the fat content of the carcass with a diminution in the water content.* These changes are in contrast with those obtained with growth hormone. In view of the fact that adrenocorticotropic hormone is known to be a growth inhibitor, these opposite alterations in the body composition after treatment with these two hormones are not surprising.

C. SUMMARY

Hypophysectomized rats, fed a limited quantity of food, were treated with either growth or adrenocorticotropic hormone. Analysis of the whole carcass showed that the growth hormone caused an increase in the protein and water content with a lowering of the fat content. On the other hand, the adrenocorticotropic hormone treatment resulted in a decrease in the water content and a gain in the fat content of the whole body.

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RENAL EXCRETION OF WATER AND ANTIDIURETIC SUBSTANCES IN PATIENTS WITH HEPATIC CIRRHOSIS AND RATS WITH DIETARY LIVER INJURY[1]

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THE problem of water retention in liver disease has been approached through both clinical and animal studies. In clinical studies Aldersberg and Fox (1943) found an abnormal retention of water when water tolerance tests were performed on patients with severe parenchymatous liver disease and recently Ralli *et al.* (1945) have demonstrated antidiuretic activity in urines from patients with hepatic cirrhosis and ascites. With respect to animal studies Aldersberg and Fox (1943) observed abnormal water tolerance tests in dogs when the liver was injured by phosphorus and histamine. Shay, Kolm, and Fels (1945) also reported the occurrence of abnormal water tolerance tests in rats fed a high fat diet. Leslie and Ralli (1947) have recently found that rats fed a high fat-low protein diet showed a decreased urine output during water tolerance tests, and they observed that the urines from such rats had antidiuretic activity.

The present study is to report some observations on the antidiuretic activity of urine from patients with cirrhosis of the liver and ascites and to observe the effect of dietary liver injury in rats on water tolerance tests and the excretion of urinary antidiuretic material.

METHODS

Seven 24 hour collections of urine were obtained from four patients with hepatic cirrhosis and ascites, and from four normal subjects. Each 24 hour urine collection was prepared for antidiuretic assay by the method of Ralli *et al.* (1945).

The liver injury was produced in rats by dietary means, as follows: Male, Sprague-Dawley rats weighing 130–170 grams, were placed on a diet of 16% casein, 51% lard, 30% corn starch, 3% salt mixture, and adequate amounts

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of vitamins A, D and the vitamin B complex for 200 days, as previously described (Hall and Drill, 1948). Groups of 10 animals each received the above diet alone or plus supplements of either casein, yeast, liver extract, ventriculin, methionine, or choline. This diet without the special supplements resulted in a marked fatty change and fibrosis in the liver which could be prevented by choline or liver extract (Hall and Drill, 1948).

While on the high-fat diet (51% fat—16% casein) a 24 hour urine specimen was collected from the 10 animals of each group on the 65th and 160th day of the study. During the collection period water was allowed *ad libitum*, but no food was given. The urine from each group of 10 rats was prepared for assay of antidiuretic substance (hereafter referred to as ADS) by the same method used for the patients' urines (Ralli *et al.* 1945). The processed urine from each group of 10 rats was adjusted to a final volume of 20 cc. for assay. Water tolerance tests were made on all of the groups of animals after the 51% fat diet had been fed for 185 to 200 days. Each group was divided into subgroups of three animals each, and two water tolerance tests were made on each sub-group.

The rats with hepatic injury, induced by the 51% fat diet, were compared with normal control animals. The normal control rats received a synthetic diet similar in all respects to the high fat diet, except that the amount of fat was reduced to 6% and the corn starch increased to 75%, the casein content remaining at 16%. These control animals received the same treatment in the collection of urine for ADS assay and for water tolerance tests as described above the for animals fed the high (51%) fat diet. Such urine collections and tolerance tests were also made at the same time as those performed on the rats receiving the high fat diet. Two 24 hour collections of urine were also made from a group of 10 rats fed a laboratory chow diet for further comparison of the ADS assay.

Both the human and rat urines were assayed for antidiuretic activity by the method of Ham and Landis (1942). The urine assays were performed on stock male rats, weighing between 200 and 300 grams, and fed a laboratory chow diet. Food was removed from the cages of the assay rats four to five hours before the test, but water was permitted up to the time of the initial hydration. The animals then received an initial hydration, 2.5% of body weight, with 0.2% NaCl. Two hours later a second hydrating dose of NaCl, equivalent to 5% of their body weight was administered. The urine extracts to be tested were injected intraperitoneally, 1 cc. per 100 grams of body weight, at the time of the second hydration. A group of control rats, similarly hydrated, received the same dose of distilled water intraperitoneally. A second group of control hydrated rats received 5 milliunits of pituitrin per cc. per 100 grams of body weight intraperitoneally. Each assay was performed on groups of three animals, and urine measurements, made every 15 minutes for 3 hours, were timed from the second hydration.

The water tolerance tests were also performed on groups of three animals, using two hydrating doses of 0.2% NaCl, as described above. Urine excretions were then measured every 15 minutes for three hours following the second hydration. In the water tolerance tests normal animals fed the 16% casein—5% fat diet and others fed the laboratory chow diet served as controls for rats receiving the high-fat diet (16% casein—51% fat).

The degree of liver injury noted in the tables was determined during the

experiment by histological study of biopsy specimens, and after 200 days by both histological study and total fat analyses.

RESULTS

The urines from the patients with cirrhosis showed an antidiuretic effect while those from normal subjects did not, as illustrated in a typical assay in Figure 1. The area under each graph of urine output may be measured in square inches. The difference in urine output between the water injected controls and animals injected with urine extracts or

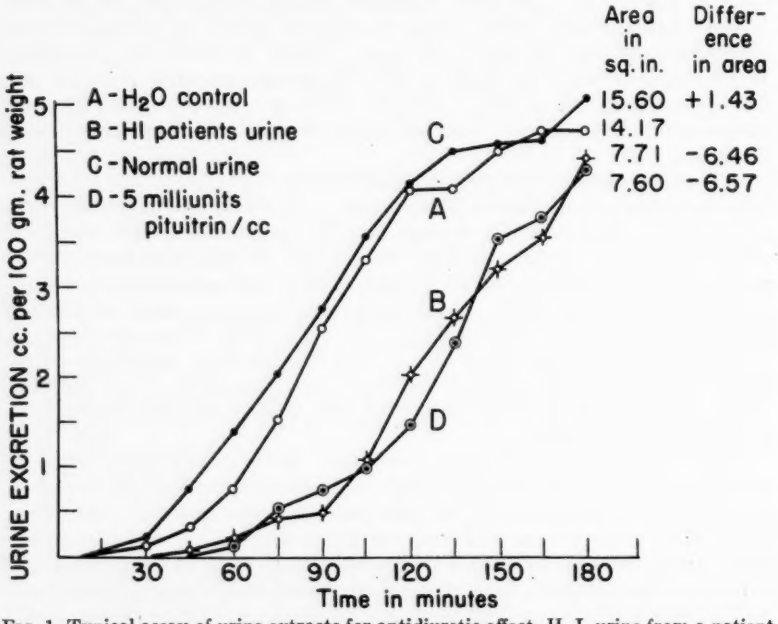

FIG. 1. Typical assay of urine extracts for antidiuretic effect. H. I. urine from a patient with hepatic cirrhosis and ascites.

pituitrin may then be recorded as a difference in square inches, a negative value indicating a lesser output of urine, and thus an antidiuretic effect of the injected material. Such assays on the human urines are summarized in Table 1. The ADS from the urine of patients with cirrhosis has an antidiuretic activity approximating that of 5 milliunits of pituitrin.

The urines from the rats fed the high-fat diet had antidiuretic activity, but an effect of the same magnitude was also obtained from the urines from animals fed the normal fat diet. Further, the antidiuretic effect was not related to the absence, presence or degree of liver injury in the different groups. The two urine samples collected from the animals fed the laboratory chow diet also showed antidiuretic activity (Table 2). The antidiuretic effect of urines from animals on

TABLE 1. ANTIDIURETIC ACTIVITY OF HUMAN URINE

Urine sample	Test No.	H_2O control in sq. inches	Pituitrin 5 mu	Urine assay
		Normal subjects		
I		11.82	−2.90	+0.48
II		16.43		−1.68
III		13.34	−7.98	−1.26
IV		14.17	−6.57	+1.43
		Patients with cirrhosis and ascites		
I*	1	11.82	−2.90	−4.54
	2	12.20	−5.65	−2.91
	3	17.45	−7.90	−7.90
II*	1	17.45	−7.90	−5.14
	2	12.60	−7.26	−5.06
	3	13.94	−5.59	−3.10
III*	1	17.40	−6.45	−5.80
	2	17.40	−6.45	−6.86
	3	19.86	−11.60	−13.96
IV*	1	14.17	−6.57	−7.72
V	1	17.40	−6.45	−4.95
VI	1	17.80		−3.79
VII	1	17.25		−4.20

* Samples from the same patient.

TABLE 2. ANTIDIURETIC ACTIVITY OF RAT URINE

Test No.	1	2	3	4	5	6
Urine sample	65th day	65th day	160th day	160th day		
H_2O Control	7.37	10.58	14.95	17.09	11.57	18.35
			Groups with no hepatic disease			
N.F. Diet		−7.34		−14.92		
H.F. + Liver Ext.		−8.33		− 8.73		
H.F. + Choline		−4.87	−12.48			
			Groups with markedly fatty livers and fibrosis			
H.F. alone	+0.84			−6.45		
H.F. + Ventriculin	−1.44		−11.91			
			Groups with intermediate stages of fatty livers			
H.F. + Casein	−0.62		− 9.91			
H.F. + Yeast	+1.41			−12.49		
H.F. + Methionine		−3.69	−10.10			
			Animals on laboratory chow diet			
					−5.17	−10.68

N.F. = normal fat diet (6% fat—16% casein).
H.F. = high fat diet (51% fat—16% casein).
H_2O control results expressed in square inches. Other groups are recorded as difference in sq. in. from water control group.

both normal and high-fat diets was not significantly altered when the urines were concentrated further or if they were diluted to their original volume of collection, or if the sediment was removed. Dialysis of the urines for 24 hrs did not remove the antidiuretic substance.

The tolerance tests also failed to show any difference in water retention between those animals on a high-fat diet, even though marked hepatic changes were produced, and animals fed a normal-fat diet (Table 3). The results in Table 3 are expressed in square inches in-

TABLE 3. WATER TOLERANCE TESTS ON RATS AFTER 185–200 DAYS OF A HIGH FAT DIET. RESULTS IN SQUARE INCHES

Test No.	1	2	3	4	5	6	7	Mean
Control, Chow Fed	17.17	13.83	10.95	12.33	18.58	19.42	15.57	15.41
Groups with no liver disease								
N.F.				16.21		13.85		15.03
H.F.+Liver Ext.	15.35	16.80	15.28	15.83	14.75		17.04	15.84
H.F.+Choline	12.79		13.25	14.70		11.99		13.18
Groups with markedly fatty livers and fibrosis								
H.F. alone		16.66		15.22	18.68	14.33		16.22
H.F.+Ventriculin	15.04	12.00	14.41	14.95	16.41		12.62	14.24
Groups with intermediate stages of fatty livers								
H.F.+Casein	12.84	14.22	14.54		12.43	14.20	15.51	13.96
H.F.+Yeast	15.90	13.71	15.49		13.72	16.16	15.94	15.15
H.F.+Methionine	14.53	14.79	17.10		12.84	14.03	17.67	15.16

stead of differences in square inches because the groups on the synthetic diet were more comparable to one another than to the animals fed the laboratory chow diet. Figure 2 illustrates the urine output in a typical assay. It can be seen in Figure 2 that no difference exists between the urine output of the groups even if the endpoint is taken as the time to excrete 50% of the hydrating volume of fluid. This was true of all of the tolerance tests.

DISCUSSION

The urines from the patients with cirrhosis and ascites had an antidiuretic effect which was comparable to that produced by 5 milliunits of pituitrin. This confirms the findings of Ralli *et al.* (1945) that in the presence of ascites the urines from patients with cirrhosis contain an antidiuretic substance. This antidiuretic effect was absent in the four normal urines that were tested. No attempt was made here to observe any correlation between the severity of the cirrhosis or the presence or absence of ascites and the antidiuretic potency of the urine. The patients used had advanced cirrhosis and ascites.

However, the rats with hepatic disease failed to show any abnormality of water metabolism in response to water tolerance tests or

assay for urinary ADS. The urine samples from rats with fibrotic and markedly fatty livers were antidiuretic, but no more so than urines from rats on a high fat diet in whom the liver injury was prevented by choline or liver extract, or in rats fed a low-fat diet or a labora-

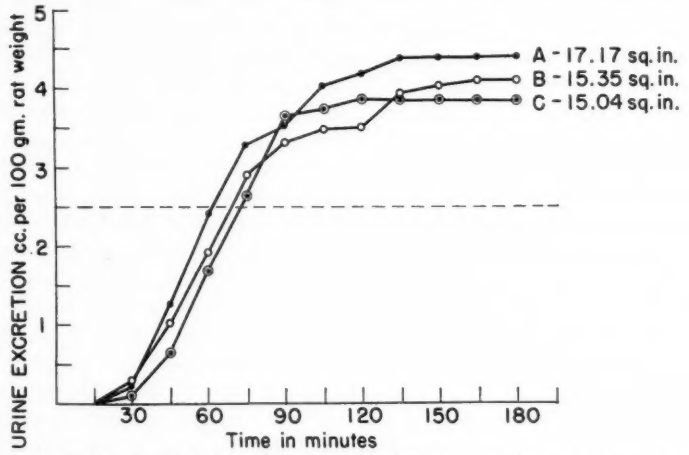

FIG. 2. Effect of a high-fat diet on water tolerance tests in rats. Curve A, water controls, chow fed. Curve B, animals fed a high-fat diet plus supplements of liver extract. Liver histology normal. Curve C, animals fed a high-fat diet. Liver showed fatty change and fibrosis. Broken horizontal indicates 50% excretion.

tory chow diet. Other investigators have found the urines from normal, well hydrated rats to be free of antidiuretic activity when assayed by methods similar to those used in the present study (Leslie and Ralli, 1947; Gilman and Goodman, 1937). The basis for this discrepancy is not clear at the present time. One other group found urines from rats fed a normal stock diet as well as those fed a high-fat diet to be free of urinary ADS (Birnie, Eversole and Gaunt, 1948).

The finding of normal tolerance tests in spite of severe hepatic injury supports the conclusions derived from the ADS determinations. Neither the high fat diet nor the fatty change and fibrosis in the liver altered the diuresis. These findings are not at variance with those of others. Shay, Kolm and Fels (1945) found that water tolerance tests were normal on animals fed a high-fat diet if they were allowed water up to the time of testing, *but were abnormal if water was withheld for 16 hours*. Dehydration alone in normal rats will produce an antidiuretic substance in rat urine (Gilman and Goodman, 1937). The animals in the present study were not dehydrated. Leslie and Ralli (1947) also withheld water (overnight) prior to the tolerance tests. They obtained the greatest retention if the diet was free of added NaCl as well as being high in fat and low in protein. Both groups of workers found abnormal tests as early as 7 days. This is very early for any marked

degree of hepatic injury by the diets used, although functional changes may have taken place. However, we found normal water tolerance tests in animals that had severe hepatic disease, who, however, were not dehydrated prior to the tolerance test. Findings similar to ours were obtained by Birnie, Eversole and Gaunt (1948). They noted that a high-fat diet did not influence the water diuresis during hydration tests. If their animals were dehydrated before the test, they obtained some indications in agreement with other workers, that fat-fed animals excreted water a little less readily than controls, but the effect was not entirely consistent.

It appears then that the fluid retention reported in rats may depend more on the state of hydration than on the presence of liver disease alone, but may be a combination of the effects of dehydration in the presence of liver disease or an abnormal diet. It is also possible that the diets used by Shay, Kolm and Fels (1945) and by Leslie and Ralli (1947) may produce somewhat different liver lesions than in the present study or may in some way make the animals more sensitive to the effects of dehydration. The increased sensitivity of rats with liver injury to the effects of dehydration seems to be the more likely possibility. As Leslie and Ralli (1947) have pointed out, such rats may also show an abnormal renal function as indicated by PSP tests, and in the presence of liver injury and/or dehydration, the renal change may be important in such studies of water tolerance and urinary antidiuretic substance.

SUMMARY

Urine extracts from patients with hepatic cirrhosis and ascites had antidiuretic activity when assayed on rats, whereas similar urine extracts from normal subjects were devoid of such activity.

Urine extracts prepared from rats with liver damage or without liver damage all showed antidiuretic activity. No difference in the intensity of the antidiuretic effect was noted in the absence or presence of liver injury.

Normal water tolerance tests were obtained in rats that had fatty and fibrotic livers. The possible relationship of any pretreatment, such as dehydration, to the tolerance test in the presence of liver injury is discussed.

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ANDROGEN:KETOSTEROID RATIOS OF RABBIT URINE[1]

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SIMULTANEOUS measurements of biologically active androgens and of 17-ketosteroids have been reported for humans and monkeys. Active androgens account for 30% to 60% of the netural ketonic fraction of human urine (Bauman and Metzger, 1940) and for about 9% to 13% of the same fraction of monkeys' urine, in terms of androsterone (Dorfman *et al.*, 1947). These figures do not represent the actual proportions in untreated urine since considerable alteration of the original steroids occurs during acid hydrolysis (Venning *et al.*, 1942).

Urinary 17-ketosteroid levels have been determined for male rabbits (Kimeldorf, 1948) and for female rabbits (De Koning *et al.*, 1948). Metabolites obtained from in vitro incubation of testosterone with rabbit liver slices have been investigated by Clark and Kochakian (1947) who could isolate neither androsterone nor its isomers from the products.

Studies on other animals show that biologically active androgens are excreted in relatively small amounts by the chimpanzee (Fish *et al.*, 1941), by the rat and dog (Clark and Kochakian, 1947), and by the bull (Hooker, 1937).

In the present study, the biologically active androgens of male and female rabbit urine extracts assayed by the chick comb method have been compared to colorimetric values of 17-ketosteroids in the same extracts.

METHODS

Three normal male and three normal female rabbits of the New Zealand strain were used. Forty-eight hour urine samples were collected over 5 cc. of concentrated hydrochloric acid, refluxed for seven minutes with concentrated hydrochloric acid (15% by volume), extracted with ether. Then the ether was washed several times with saturated sodium carbonate, with two normal sodium hydroxide, and with distilled water, to obtain the crude neutral fraction. This extract either was assayed directly or was treated with Girard's reagent T yielding the neutral ketonic fraction (Pincus, 1945).

Colorimetric analyses were run on the Beckman DU Spectophotometer

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[2] The authors wish to acknowledge the assistance of J. De Koning who prepared some of the urine extracts.

at 520 mμ., 0.02 mm. slit width, using dehydroisoandrosterone[3] as a standard. The 2.5 normal alcoholic potassium hydroxide was stabilized with ascorbic acid (Wilson and Carter, 1947). The Zimmerman reaction was carried out in absolute alcohol by the method of Talbot *et al.* (1940). All extracts and blanks were done in triplicate.

Assays for androgenic activity were performed by the chick comb method of Frank *et al.* (1942) using androsterone[3] standards. Figure 1 shows the comb weight/body weight ratios (minus control values) and amounts of

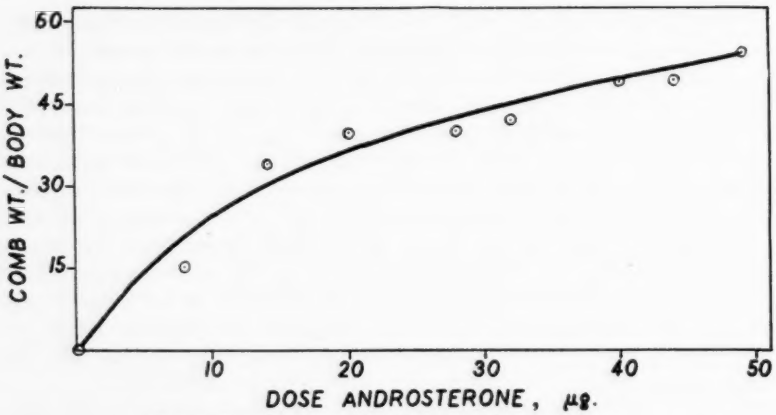

FIG. 1. Standard curve for androgen determinations.

androsterone applied topically. Ten chicks were employed for each standard value or urine extract and ten for each set of controls. A total of 790 chicks were used. Recovery experiments were performed with crystalline androsterone.

Preliminary attempts to characterize the ketosteroids involved androgenic assays of four chromatographed eluates, separations of alpha and beta ketosteroids with digitonin (Pincus, 1945) and separation of alcoholic and non-alcoholic ketosteroids by means of succinic anhydride (Pincus and Pearlman, 1941).

RESULTS AND CONCLUSIONS

An examination of the data of Tables 1 and 2 disclosed a low level of biologically active androgens. In terms of androsterone, the active material constituted an average of about 2% of the neutral ketonic fraction of male rabbit urine, and less than 1% of the same fraction of female rabbit urine. It should be pointed out that extracts from ovariectomized rabbits had about the same androgenic titer as that of the normal females.

According to the recovery experiments (Table 3), a considerable

[3] Generously supplied by the Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

TABLE 1. ANDROGENS AND 17-KETOSTEROIDS IN MALE RABBIT URINE EXTRACTS

Rabbit's age months	Urine volume cc.	Fraction	Androgens as androsterone mg.	17-ketosteroids mg.	% androgens in extract
7	425	Neutral ketonic	0.02	2.4	1
	450	Neutral ketonic	0.03	4.3	1
	155	Crude neutral	0.00	1.4	0
	480	Crude neutral	0.01	3.6	0
12	160	Neutral ketonic	0.09	2.8	3
	240	Neutral ketonic	0.12	5.8	2
	95	Crude neutral	0.04	1.5	3
	225	Crude neutral	0.01	4.2	0
16	205	Neutral ketonic	0.12	2.4	5
	385	Neutral ketonic	0.05	2.8	2
	290	Crude neutral	0.01	3.2	0
(Average male)	310	Neutral ketonic	0.07	3.4	2

proportion of any androsterone present in the urine would be expected to form biologically inactive ketosteroids during hydrolysis and extraction. (Likewise, Venning *et al.* in 1942 found that androsterone sulfate is hydrolyzed largely to inactive steroids such as androstenone-17.) Accordingly, the proportion of active androgens in raw rabbits' urine is probably higher than that of the urine extracts but still lower than the androgenic titer of human urine. As an additional check on the techniques, two human urine extracts prepared in the same manner were tested by the chick comb method and were found to contain about nine times as much androgenic activity as the rabbit extracts.

TABLE 2. ANDROGENS AND 17-KETOSTEROIDS IN FEMALE RABBIT URINE EXTRACTS

Rabbit's age months	Urine volume cc.	Fraction	Androgens as androsterone mg.	17-ketosteroids mg.	% androgens in extract
5	340		−0.05	2.3	−2
		Four chromatographed eluates	−0.05	total before chromatography	−2
			0.02		1
			−0.02		−1
8	150	Neutral ketonic	−0.01	4.0	0
	300	Neutral ketonic	−0.01	3.9	0
	370	Neutral ketonic	−0.01	2.8	0
	400	Neutral ketonic	0.00	4.7	0
	360	Neutral ketonic	0.03	4.5	1
10	150	Neutral ketonic	0.04	1.4	3
	375	Neutral ketonic	0.02	2.8	1
	1000	Neutral ketonic	0.03	8.0	0
	390	Crude neutral	0.00	2.5	0
	500	Crude neutral	−0.01	3.3	0
(Average female)	390	Neutral ketonic	0.01	4.0	1

TABLE 3. RECOVERY OF ANDROSTERONE IN RABBIT URINE EXTRACTS

Androsterone mg.	Added to	17-ketosteroids in unspiked aliquot mg.	17-ketosteroids in spiked aliquot mg.	Androgens in unspiked aliquot mg.	Androgens in spiked aliquot mg.	Recovery of 17-ketosteroids %	Recovery of androsterone %
3.6	Neutral ketonic fraction	5.0	8.6	0.03	3.69	—	103
1.4	Neutral ketonic fraction	4.1	5.5	0.02	1.32	—	93
1.4	Neutral ketonic fraction	3.5	4.9	0.01	1.42	—	99
1.4	Neutral ketonic fraction	4.1	5.5	0.02	1.52	—	116
3.1	Urine before hydrolysis	2.8	6.0	0.04	1.35	102	42
3.1	Urine before hydrolysis	2.6	5.5	0.00	1.07	94	34
3.1	Urine before hydrolysis	3.2	5.7	0.12	1.05	82	30

Since the beta-ketosteroid fraction was not present in detectable amounts (Table 4), neither dehydroisoandrosterone nor isoandrosterone apparently are excreted in appreciable quantities by normal rabbits. (Similarly, no digitonin-precipitable ketosteroids were found in normal guinea pig urine by Miller and Dorfman, 1945.)

The proportions of alcoholic and non-alcoholic ketosteroids (Table 4) do not represent those originally present in the urine since acid

TABLE 4. ALPHA & BETA, OH & NON-OH KETOSTEROIDS IN MALE RABBIT URINE EXTRACTS

Rabbit's age months	Urine volume cc.	Alpha-ketosteroids mg.	Beta-ketosteroids mg.	Alcoholic ketosteroids mg.	Non-alcoholic ketosteroids mg.
7	470	2.9	0	—	—
	535	2.9	0	—	—
	525	—	—	0.89	0.63
12	255	3.2	0	—	—
	300	3.6	0	—	—
	225	—	—	0.68	0.75
16	470	2.9	0	—	—
	500	2.1	0	—	—
	400	—	—	0.78	0.66

hydrolysis causes a large conversion of alcoholic ketosteroids to non-alcoholic ketosteroids (Pincus and Pearlman, 1941).

The exact chemical nature of the neutral 17-ketosteroids excreted by rabbits must await their separation and identification.

SUMMARY

Low androgenic titers are found in rabbit urine extracts. In terms of androsterone, only about 2% of the neutral ketonic fraction (less than 1% of female) rabbit urine is biologically active. Recovery experiments and comparative data indicate that the proportion of androgens in untreated rabbits' urine, while probably considerably

higher than that of the extracted material, is nevertheless but a fraction of the activity of androgens in human urine extracts. Both alcoholic and non-alcoholic ketosteroids are present in the neutral ketonic fraction. The presence of beta-ketosteroids has not been detected.

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CHANGES IN THE DISTRIBUTION AND CONCENTRATION OF ALKALINE PHOSPHATASES IN TISSUES OF THE RAT AFTER HYPOPHYSECTOMY OR GONADECTOMY, AND AFTER REPLACEMENT THERAPY[1]

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IT IS becoming increasingly evident that hormones exert their regulatory effects on tissues and organs by influencing the rate of enzymatic processes. Hertz (1945) has shown that estrogen requires the presence of folic acid, now regarded as a prosthetic group of an enzyme, to produce growth of the chick's oviduct. Biddulph, Meyer and McShan (1946) have demonstrated changes in the concentration of succinic dehydrogenase and other enzymes in the ovaries of rats during different stages of the reproductive cycle. Huggins and Russell (1946) investigated the effects of various hormonal conditions upon the concentration of phosphatases in the prostate gland. Fluctuations in the alkaline phosphatase activity of the uterus have been correlated with the reproductive cycle in mice and in primates (Atkinson and Engle, 1947; Atkinson and Elftman, 1947).

Cytochemical studies in this laboratory have also revealed, as incidental observations, that phosphatases vary in different physiological states. The organs studied have included the uterus (Wislocki and Dempsey, 1945), thyroid (Dempsey and Singer, 1946), mammary gland (Dempsey, Bunting and Wislocki, 1947), and placenta (Dempsey and Wislocki, 1947). Since all of these organs are controlled by hormones, it seemed desirable to study directly the effects of endocrine stimulation on phosphatase activity in a number of end-organs. To this end, we have studied the cytochemical distribution of the alkaline phosphatases capable of dephosphorylating glycerophosphate, yeast nucleic acid, and fructose diphosphate in selected organs and tissues of rats after hypophysectomy or gonadectomy, and after replacement therapy.

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EXPERIMENTAL PLAN

Young adult male and female rats of the Sprague-Dawley strain were used. Fourteen were hypophysectomized, of which four females and two males were killed after various intervals without further treatment. A month after operation, one male and one female were given daily subcutaneous injections for a week of a powder made from rat pituitaries dried in acetone. Another male was injected daily with androgen and a female with estrogen. Two male and two female rats were gonadectomized. One of each sex was killed at the end of a month without further treatment. The other castrated male was injected with androgen and the female with estrogen. One normal male and one untreated female rat were used as controls. The details of the experimental plan are shown in the accompanying table.

The animals were killed with chloroform. From the control rats, those which were hypophysectomized, and those given pituitary powder the following organs were taken: testis and seminal vesicle, or ovary, uterus and mammary gland; a segment of the trachea with the adjoining thyroid, parathyroid and muscles; adrenal gland; kidney; pancreas; duodenum; and submaxillary gland. From the gonadectomized animals and those given steroid hormones the seminal vesicle or uterus and mammary gland were taken. The organs were fixed in chilled 80 per cent alcohol. Paraffin sections were prepared and stained by a modification of Gomori's alkaline phosphatase method (Dempsey and Deane, 1946). Glycerophosphate, fructose diphosphate and yeast nucleic acid were used as substrates.[2] The solutions were buffered to pH 9.5, and the preparations were incubated in each for 3, 6 and 24 hours at 37°C. The nuclei were counterstained lightly with picrocarmine.

RESULTS

In most of our observations, similar results were obtained with all three substrates.

Thyroid. The location and relative concentration of alkaline phosphatase in the thyroid were found to be similar when tested with the three different substrates, but the intensity of the reaction varied greatly. The heaviest deposits were observed after fructose diphosphate and nucleic acid, whereas only faint reactions occurred when glycerophosphate was employed. The most intense activity occurred in the endothelium of the capillaries and in the cytoplasm and nuclei of the large follicles at the periphery of the gland (Fig. 1). Lesser reactions occurred in the cytoplasm and nuclei of the central follicles, the smallest of which were entirely unreactive.

After hypophysectomy phosphatase disappeared from the endothelium within the thyroid, while that of the cytoplasm and nuclei of the follicular cells remained. Indeed, due perhaps to the shrinking of the cells after hypophysectomy, the enzyme appeared more concentrated in the cytoplasm of the parenchymal cells and was no longer re-

[2] Yeast nucleic acid and fructose diphosphate were obtained from the Schwarz Laboratories, 202 E. 44th St., New York City. The glycerophosphate was a mixture of the alpha and beta forms and was purchased from the J. T. Baker Chemical Co., Philadelphia.

stricted to peripheral follicles but occurred in the central follicles as well (Fig. 2).

After replacement therapy with whole pituitary powder, phosphatase reappeared in the capillary endothelium. Concomitantly, as the follicular epithelium became taller, its cytoplasmic phosphatase declined so that the total condition approximated normal.

Adrenal cortex. The distribution of alkaline phosphatase in the adrenal cortex exhibits a sex difference in the rat. Much more of the enzyme is present in the male than in the female. Elftman (1947) observed this difference between male and female Swiss mice, in which only traces are present in the cortex of the female, whereas considerable quantities occur in that of the male. In rats, the difference between males and females is similar but not so marked. In normal females, considerable quantities of phosphatase occurred in the zona glomerulosa, where the endothelium of the sinusoids stained quite markedly, the nuclei of the parenchymal cells moderately and the cytoplasm faintly. Only traces occurred in the cells of the fasciculata and reticularis, but detectable quantities of enzyme were displayed in the endothelium of the sinusoids as is shown in Fig. 3. These reactions were best elicited with fructose diphosphate and nucleic acid, but similar though fainter distributions were seen with glycerophosphate.

In the male rat, the glomerulosa stained identically with that of the female. Beneath the glomerulosa a narrow transitional region occurred which was deficient in phosphatase. The fasciculata and reticularis contained abundant quantities of enzyme located in the nuclei and cytoplasm of the parenchyma and in the endothelium of the

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EXPLANATION OF FIGURES ON PLATE 1

All photomicrographs are of sections incubated at 37°C. in phosphate substrates buffered to pH 9.5. Nuclei lightly counterstained with paracarmine. All figures except of pancreas and mammary gland ×200.

FIG. 1. Fructose diphosphatase reaction at periphery of the thyroid gland of a normal rat. The enzyme is located principally in the endothelial cells of the capillaries and in the nuclei of the follicular cells. Some reaction occurs also in the cytoplasm of the epithelium of the outer follicles. In the upper portion of the picture the capillaries of the adjacent striated muscle may be seen to be strongly reactive. Incubation time, 6 hours.

FIG. 2. Fructose diphosphatase in the thyroid gland of a rat hypophysectomized 34 days previously. The enzyme has disappeared from the capillary endothelium but persists in the walls of the two small arteries seen in the upper right corner of the picture. The enzyme is still active in the greatly flattened nuclei as well as in the cytoplasm of the follicular cells. Incubation time, 6 hours.

FIG. 3. Fructose diphosphatase in the adrenal cortex of a female rat hypophysectomized 26 days previously. The strong reaction in the glomerulosa and of the endothelium lining the sinusoids of the inner zones is essentially normal, but a notable loss of enzyme from the fascicular cells has occurred. Incubation time, 3 hours.

FIG. 4. Nucleic acid phosphatase in the adrenal cortex of a female rat hypophysectomized 120 days previously. Essentially comparable reactions were obtained with the other substrates. The enzyme has disappeared completely from the deeper layers of the cortex but has persisted in the endothelium lining the sinusoids of the zona glomerulosa. Incubation time, 6 hours.

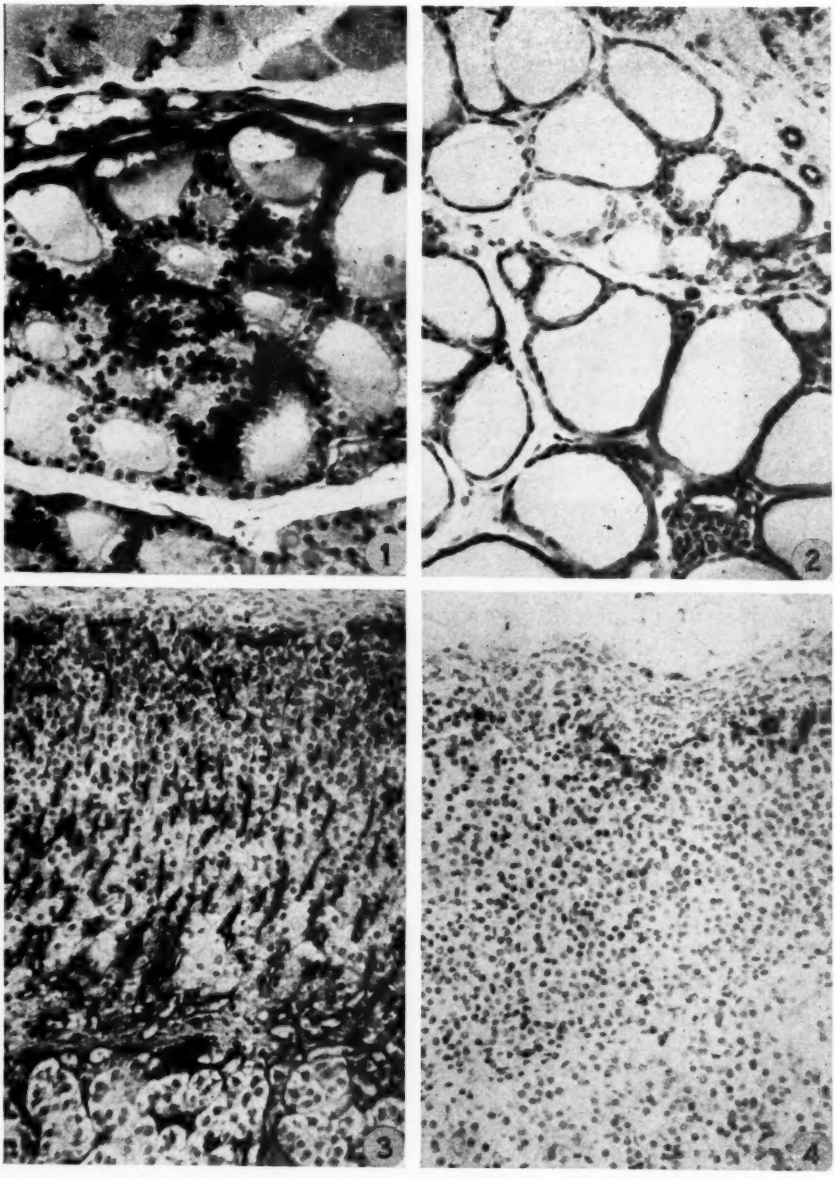

PLATE 1

sinusoids. In this sex also, the reactions were more intense with fructose diphosphate and nucleic acid than with glycerophosphate. The sex difference therefore consists in the location of considerably greater quantities of enzyme in the cells of the deeper zones of the cortex in the male than in the female.

After hypophysectomy, the zona glomerulosa retained its phosphatase in both male and female rats. The endothelium of the sinusoids stained as intensely as in normal rats for as long as four months after the operation. The intracellular phosphatase decreased somewhat but was still perceptible (Fig. 4).

Contrary to its behavior in the glomerulosa, the phosphatase of the fasciculata and reticularis decreased notably or disappeared after

TABLE 1. TREATMENT OF INDIVIDUAL RATS STUDIED FOR ALKALINE PHOSPHATASES

Operation	Hormone treatment (days after operation)	Date of autopsy after operation
	Females	
—	—	—
Hypophysectomy	—	26
Hypophysectomy	—	34
Hypophysectomy	—	120
Hypophysectomy	—	120
Hypophysectomy	pituitary powder[1] (26–33 d.)	34
Hypophysectomy	estrogen[2] (26–33 d.)	34
Ovariectomy	—	26
Ovariectomy	estrogen (26–33 d.)	34
	Males	
—	—	—
Hypophysectomy	—	26
Hypophysectomy	—	34
Hypophysectomy	pituitary (26–33 d.)	34
Hypophysectomy	androgen[3] (26–33 d.)	34
Castration	—	26
Castration	androgen (26–33 d.)	34

[1] 2½ rat pituitaries daily (acetone-dried powder, in saline).
[2] 10 rat units daily (Progynon B, Schering, in oil).
[3] 2½ mg. daily (Testosterone propionate, Schering, in oil).

hypophysectomy. In the female, the endothelial phosphatase persisted for one month (Fig. 3) but disappeared by the fourth month after the operation (Fig. 4). In the male, the phosphatase-free transitional region between the glomerulosa and fasciculata became wider and, as time elapsed, the enzyme gradually disappeared from the deeper zones, although moderate amounts persisted in the reticularis for at least five weeks.

Replacement therapy with whole pituitary powder caused a reappearence of phosphatase to a condition approximating that of the normal gland.

Testis. Alkaline phosphatase occurs in both the tubules and the

intertubular tissue in the testis of the normal rat (Fig. 5) and may be revealed with almost equal ease by fructose diphosphate, nucleic acid or glycerophosphate. In the tubules, the most prominent reactions occur in the spermatids and in a zone of almost hair-line sharpness in the basement membranes surrounding the tubules. Flattened cells frequently appear in this membrane; their elongated nuclei are darker than the cytoplasm. In places, this basement membrane is split into a double lamina separated by a clear space. Finally, among the tubular elements the nuclei of the spermatogonia and the chromosomes of the spermatocytes exhibit moderate reactions.

The intertubular tissue also exhibited variable amounts of alkaline phosphatase. Most prominent are the endothelial cells lining the blood vessels. The endothelium of arteries, veins, the smaller arterioles and venules, and capillaries was almost equally reactive. The interstitial cells were diffusely but faintly stained. Although the intertubular cells are a mixture of Leydig's cells and connective tissue elements, no discrimination among them was possible by the phosphatase reaction since all stained in the same faint fashion.

After hypophysectomy, the testis of the rat became entirely negative for alkaline phosphatase (Fig. 6). After replacement therapy with whole pituitary, the enzyme returned to its original distribution and intensity in all elements.

Ovary. Alkaline phosphatase occurred ubiquitously in the ovary of the normal rat. It was demonstrable in the graafian follicles, corpora lutea, interstitial tissue and blood vessels. In follicles, from small ones with a newly formed antrum to the largest, phosphatase activity was high in the endothelium of the vascular wreath situated in the theca interna. Moderate reactions also occurred in the nuclei and diffusely in the cytoplasm of the thecal cells. After incubation periods of 3 to 6 hours the granulosa cells usually were negative, but, after 24 hours, moderate reactions could be seen there too, especially in the nuclei. During atresia, the vessels and cells of the theca retained their enzyme content, whereas that of the granulosa was lost as these cells degenerated. In the corpus luteum, the endothelium of the sinusoids was strongly positive. The first and second generations of corpora lutea contained faint but appreciable quantities of phosphatase in the nuclei and cytoplasm of the luteal cells. Older corpora and the interstitial tissue, which represent the remnants of luteal tissue and of thecal tissue from atretic follicles, also contained strong concentrations of phosphatase in their blood vessels but cellular reactions were faint or absent.

One month after hypophysectomy, no large follicles were to be found, but small ones with beginning antrum-formation exhibited strong vascular reactions in the thecal wreath (Fig. 10). Similarly, the endothelium of other blood vessels continued to exhibit phosphatase, whether located in large vessels in the medulla of the organ or in

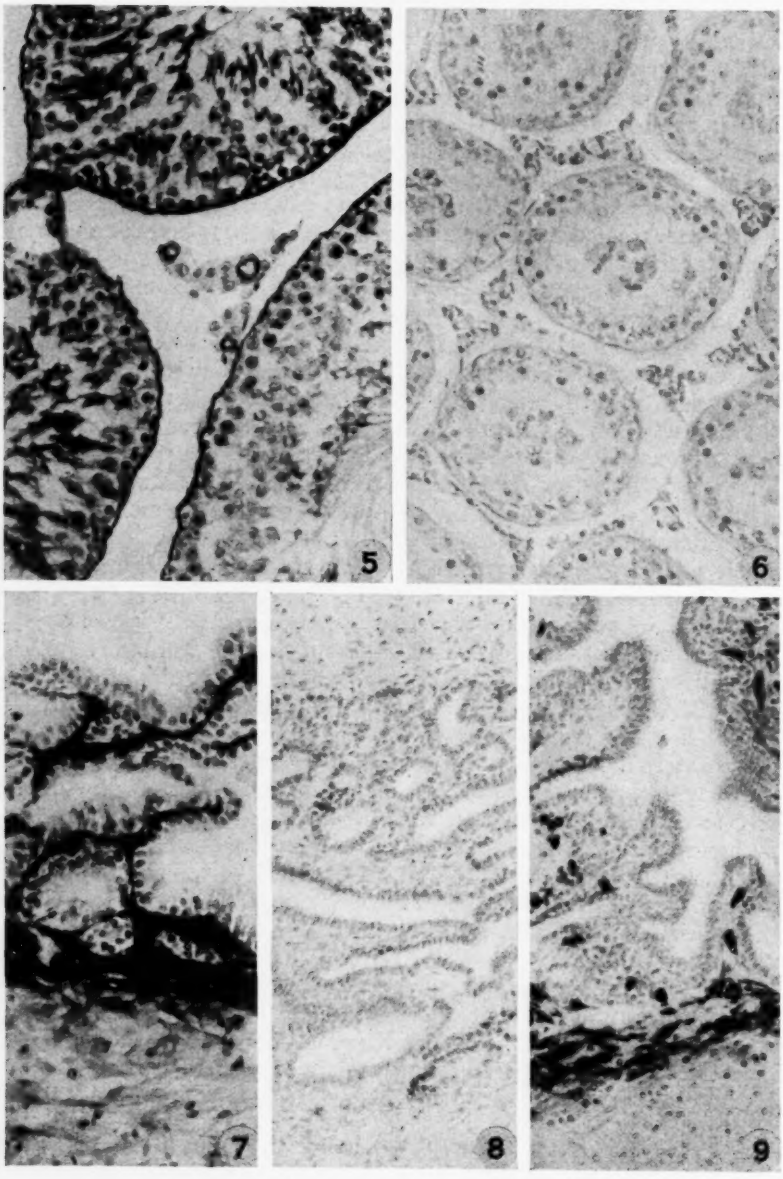

PLATE 2

small ones in corpora lutea and the interstitial tissue. On the other hand, the cellular reactions were noticeably decreased or absent. Four months after hypophysectomy the phosphatase content of the endothelium was still present but noticeably diminished (Fig. 12). Thus, in the corpora lutea, which in the rat persist for long times after hypophysectomy, the initial change is a decrease of the phosphatase of the parenchymal cells. The endothelially located enzyme also declines but only slowly over a long period.

Replacement therapy with whole pituitary restored the phosphatase of the follicles and newly formed corpora to their normal appearance (Fig. 11). Older corpora lutea were not perceptibly affected by the treatment.

Pancreas. The distribution of phosphatase in the pancreas has been described recently by Deane (1947). In agreement with her findings, we have observed alkaline phosphatase in the endothelium lining the small blood vessels and in the stroma of the larger ducts. After incubation in nucleic acid, an intense reaction occurs in the nuclei and in the basal part of the acinar cells—the location of the basal ergastoplasm. Very little enzyme activity is seen with glycerophosphate as substrate. Moreover, in the rat, two types of cells may be distinguished in the islets of Langerhans. In one of these, constituting cells located largely at the periphery of the islets, an intense reaction was demonstrable in both the nuclei and cytoplasm, especially following the use of either fructose diphosphate or nucleic acid. In the other, centrally located cells, the nuclei were moderately active, but the cytoplasm stained only weakly (Fig. 16). The intensely staining outer group of cells correspond in location to the alpha cells, whereas in the rat the beta cells are centrally located and coincide in location with those exhibiting the weak phosphatase reaction. Interestingly enough, this difference in activity of the two types of islet cells is far more extreme in the rat than in other species, since we have observed slight or no differences in the islet cells of the monkey and man.

EXPLANATION FOR FIGURES ON PLATE 2

FIG. 5. Fructose diphosphatase reaction in the testis of a normal rat. The reaction is strongest in the basement membrane and spermatids, moderate in the endothelium and nuclei of the spermatozoa and spermatocytes, and weak in the cytoplasm of the cells and in the interstitial cells. Incubation time, 6 hours.

FIG. 6. Fructose diphosphatase in the testis of a rat hypophysectomized 26 days previously. The enzyme has disappeared completely. Incubation time, 6 hours.

FIG. 7. Fructose diphosphatase in the seminal vesicle of a normal rat. Strong concentrations of the enzyme may be seen in the reticulum and blood vessels underlying the epithelium. Incubation time, 6 hours.

FIG. 8. Glycerophosphatase in the seminal vesicle of a rat hypophysectomized 26 days previously. The enzyme has disappeared entirely. Incubation time, 6 hours.

FIG. 9. Fructose diphosphatase in the seminal vesicle from the same animal shown in Fig. 8. Although glycerophosphatase has entirely disappeared by this time, the blood vessels still show a notable enzyme activity when tested with fructose diphosphate. Incubation time, 6 hours.

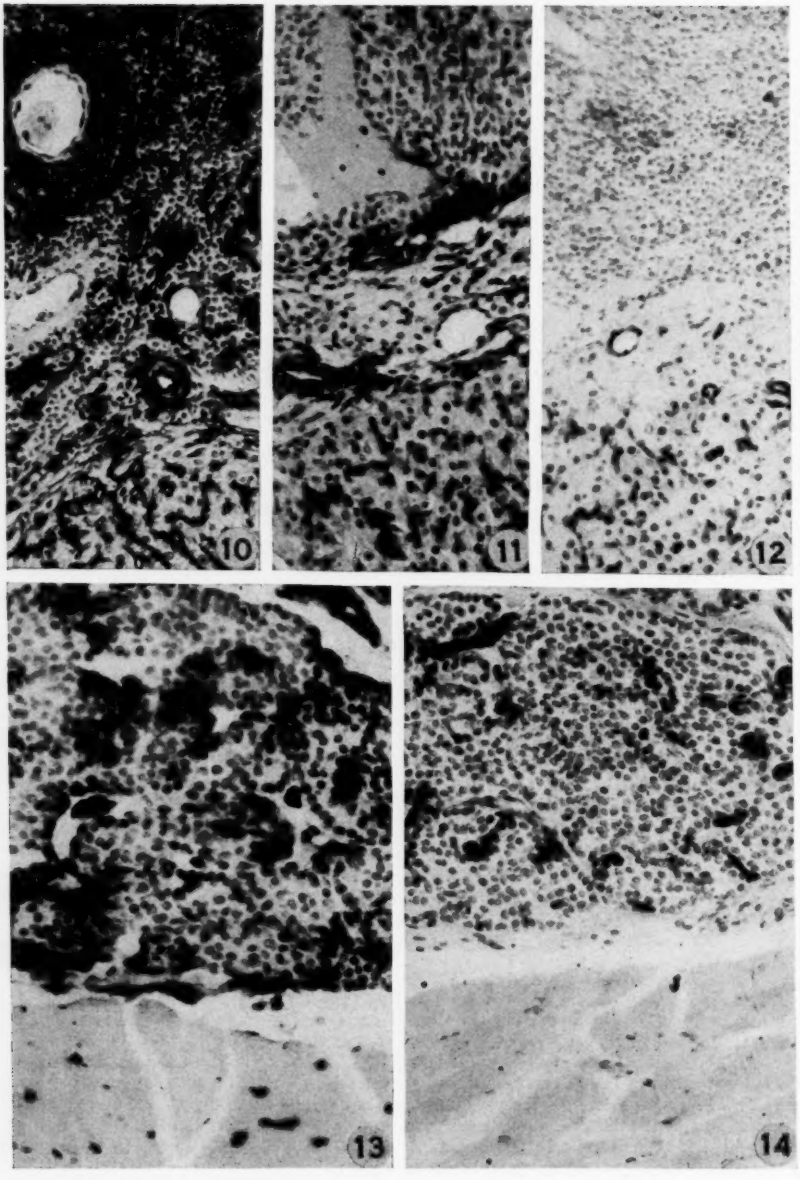

PLATE 3

After hypophysectomy, the intensity of the phosphatase reaction declined in both the acinar and islet portions of the pancreas. The distribution remained unaffected, however, and the response of the gland is therefore unlike that of most of the endocrine organs which are under direct control by the hypophysis.

Parathyroid. Alkaline phosphatase was demonstrated easily in the endothelial cells of the parathyroid capillaries and in the nuclei of the parenchymal cells (Fig. 13). Fructose diphosphate yielded the most intense reactions, those produced with nucleic acid and glycerophosphate being similar in distribution but weaker in intensity. After hypophysectomy, the intensity of the reaction declined perceptibly, but no change in distribution could be detected (Fig. 14).

Seminal vesicle. Intense phosphatase activity was demonstrable in the stroma and capillaries immediately underneath the epithelium of the seminal vesicle (Fig. 7). In the normal rat, these deposits of enzyme may be visualized equally well by employing any of the substrates used in this study. After hypophysectomy, the phosphatase activity declined and disappeared, but the rate of disappearance varied with the different substrates. One month after hypophysectomy, the seminal vesicle contained no glycerophosphatase (Fig. 8) but exhibited a considerable activity when incubated in fructose diphosphate (Fig. 9). Similar results followed gonadectomy. Restoration of the normal complement of enzyme was accomplished by injecting pitui-

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EXPLANATION OF FIGURES ON PLATE 3

FIG. 10. Fructose diphosphatase in the ovary of a rat hypophysectomized 26 days previously. In the lower right-hand corner, the edge of a corpus luteum may be seen. In it strong phosphatase activity occurs in the endothelium of the sinusoids. In the upper left-hand corner a small atretic follicle exhibits intense activity in the rete of vessels surrounding the follicle. The nuclei in the interstitial tissue are also positive. In the normal gland the cytoplasmic reactions are more intense. Incubation time, 3 hours.

FIG. 11. Nucleic acid phosphatase in the ovary of a rat hypophysectomized 34 days previously and injected with whole pituitary powder. In the upper portion of the figure is the wall of a graafian follicle with a strong concentration of phosphatase in the region of the theca interna. The lower portion of the figure illustrates a corpus luteum in which the sinusoids exhibit strong phosphatase activity. The luteal cells also react more strongly than in Fig. 10. Incubation time, 3 hours.

FIG. 12. Nucleic acid phosphatase in the ovary of rat hypophysectomized 120 days previously. The lower portion of the figure illustrates a corpus luteum in which the sinusoids still contain a detectable phosphatase activity. No activity remaining in interstitial tissue. Incubation time, 3 hours.

FIG. 13. Fructose diphosphatase activity in the parathyroid gland of a normal rat. The endothelium of the capillaries within the gland is strongly reactive, and underneath the gland the capillaries of the adjacent striated muscle also react positively. Incubation time, 3 hours.

FIG. 14. Fructose diphosphatase in the parathyroid gland of a rat hypophysectomized 34 days previously. In the gland itself phosphatase is still present but is reduced somewhat in amount as compared with the normal, illustrated in Fig. 12. The capillaries of the striated muscle shown at the bottom of the figure have completely lost their phosphatase reaction. Incubation time, 3 hours.

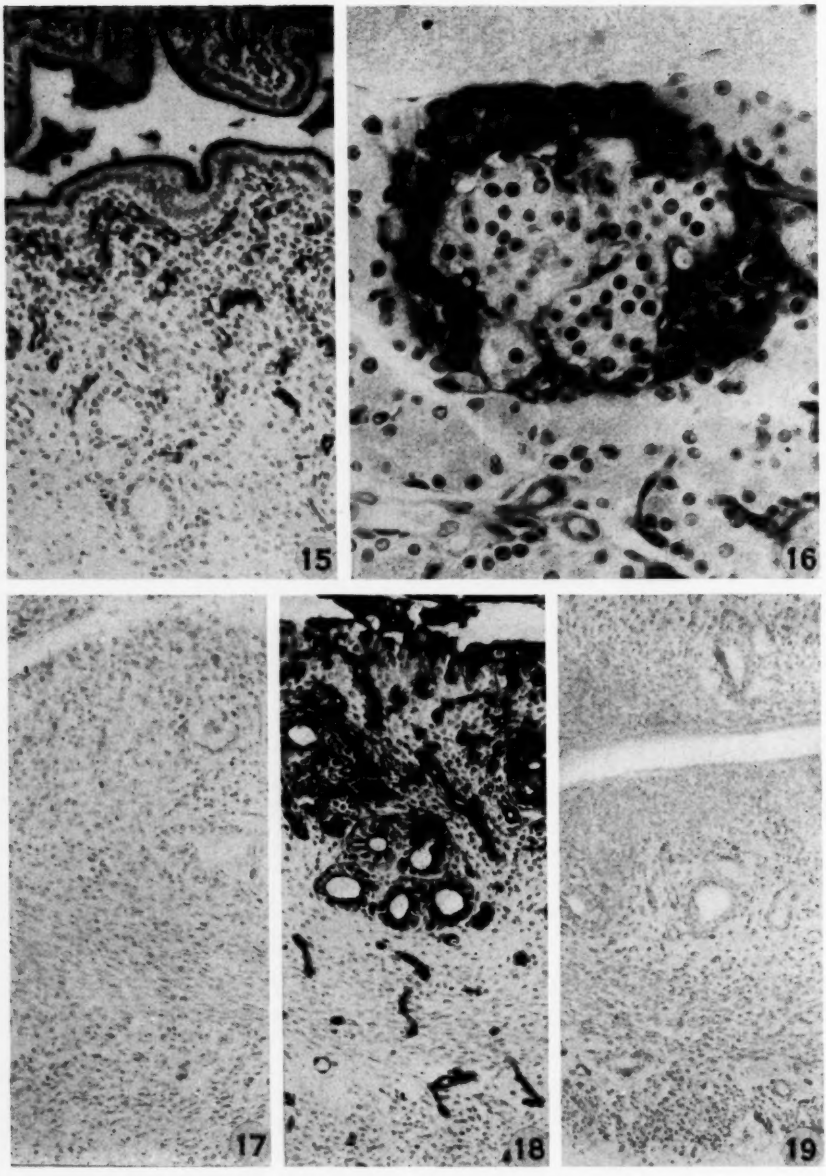

PLATE 4

tary powder or testosterone into hypophysectomized or castrated rats, respectively.

Uterus. The normal distribution of alkaline glycerophosphatase in the uterus has been studied by Atkinson and Elftman (1947) and Atkinson and Engle (1947). The enzyme occurs normally in the lumen, mucosa, stroma and capillary endothelium. These authors have also described the disappearance of phosphatase after ovariectomy and its restoration on replacement therapy with estrogens. Except for minor differences in distribution in the different species, our results are in complete agreement with theirs. We can add to their findings the observation that glycerophsophatase also disappears rapidly from the uterus after hypophysectomy and is restored after replacement therapy with pituitary powder. When fructose diphosphate was used as a substrate, the phosphatase persisted much longer after hypophysectomy or ovariectomy than when glycerophosphate was employed. Fig. 17 illustrates a negative glycerophosphatase reaction in the uterus of a rat hypophysectomized 26 days previously. The positive fructose diphosphatase reaction of the same uterus is illustrated in Fig. 18. The fructose diphosphatase ultimately disappeared, however, as is shown in Fig. 19, which illustrates a negative reaction in the uterus from a rat hypophysectomized 4 months previously. Thus, both glycerophosphatase and fructose diphosphatase disappear from the uterus following withdrawal of the hormonal stimulus to the organ; but whereas glycerophosphatase disappears rapidly, fructose diphosphatase persists for a longer time.

As described by Atkinson and coworkers, phosphatase activity is restored to all sites in ovariectomized animals given estrogen. It is likewise restored in hypophysectomized animals given estrogen or pituitary powder (Fig. 15).

EXPLANATION OF FIGURES ON PLATE 4

FIG. 15. Nucleic acid phosphatase in the uterus of a rat hypophysectomized 34 days previously and injected with whole pituitary powder. The location and concentration of phosphatase are essentially normal in this figure. Incubation time, 6 hours.

FIG. 16. Fructose diphosphatase in an islet of Langerhans in the pancreas of a rat hypophysectomized 34 days previously. Notable concentrations of the enzyme can be seen in the capillaries of the exocrine portion of the pancreas and in the nuclei and cytoplasm of the islet cells. The cells in the peripheral portion of the islet are much more strongly positive than those in the center. The peripheral cells correspond in location to the alpha cells, whereas the central cells are of the beta variety. Incubation time, 24 hours, ×500.

FIG. 17. Glycerophosphatase in the uterus of a rat hypophysectomized 26 day previously. Normally, this enzyme has a distribution similar to that illustrated in Figure 15. It has completely disappeared after hypophysectomy. Incubation time, 6 hours.

FIG. 18 Fructose diphosphatase in the uterus of the same animal shown in Fig. 17. With this substrate, enzyme activity persists for at least 26 days after hypophysectomy. Incubation time, 3 hours.

FIG. 19. Fructose diphosphatase in the uterus of a rat hypophysectomized 120 days previously. By this time fructose diphosphatase has disappeared entirely from the uterus. Incubation time, 6 hours.

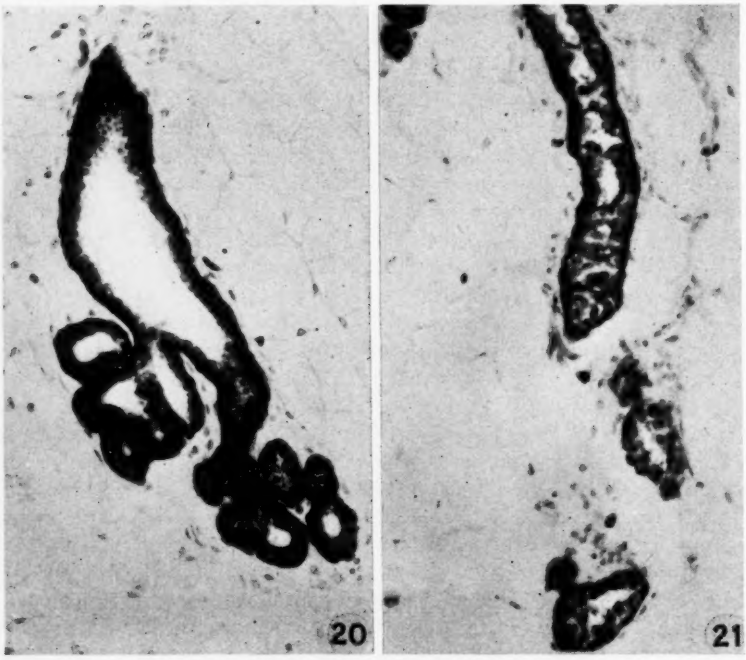

FIG. 20. Nucleic acid phosphatase in the mammary gland of an estrous rat. Incubation time, 6 hours ×240.

FIG. 21. Nucleic acid phosphatase in the mammary gland of a rat hypophysectomized 120 days previously. Incubation time, 6 hours ×240.

Mammary gland. Dempsey, Bunting and Wislocki (1947) have recently described the location of phosphatase in the rat's mammary gland. This organ exhibits enzymatic activity in both the alveolar epithelium and in the curious basket, or myoepithelial, cells which surround the alveoli and ducts (Fig. 20). In pregnancy and during lactation the phosphatase activity declines in the epithelium; heavy deposits are restricted to the greatly attenuated basket cells and to the capillaries.

After either hypophysectomy or gonadectomy, phosphatase persisted in the mammary glands of rats, albeit in reduced amounts (Fig. 21). The enzyme activity of this organ therefore does not depend completely upon the hormonal stimuli emanating from the hypophysis or ovaries.

Striated muscle. Fig. 13 illustrates the phosphatase content of the endothelium lining the capillaries of the pretracheal muscles adjacent to the parathyroid gland. The endothelial phosphatase was detectable in sections incubated in any of the substrates, but was most prominent with fructose diphosphate. After hypophysectomy, the phospha-

tase quickly disappeared from the endothelium (Fig. 14). Replacement therapy with whole pituitary effected a prompt restoration of the normal appearance.

Submaxillary gland, kidney and duodenum. The normal distribution of alkaline phosphatases has been described earlier for the salivary gland (Deane, 1947), the kidney (Deane and Dempsey, 1945) and the duodenum (Dempsey and Deane, 1946). Following hypophysectomy all of these organs experienced a reduction in over-all activity similar to that of the parathyroid. Again, no change could be detected in the sites in which the enzyme was located.

DISCUSSION

Modern endocrinology has developed largely from the application of a small number of simple procedures. A hormone is defined as a substance produced in one region and transported by the blood stream to another part of the body where it exerts its effect. Consequently, the principal procedure of endocrinology consists in the injection of extracts coupled with examination of selected end-organs to determine whether or not changes occur in them. In a large fraction of instances, this examination has determined only the changes in size or microscopic appearance which follow endocrine stimulation. As a result, we know today that many endocrine factors exist and that they selectively stimulate various specific organs, but we have next to no knowledge about the mechanisms by which the hormones exert their effects.

The comparatively recent recognition that biochemical conversions are controlled by enzymes having no counterparts in non-living systems forces attention upon the relationships of the hormones to these regulatory mechanisms. For example, biological oxidations do not occur simply upon exposure of the substrates to atmospheric or nascent oxygen, but require catalysis by a complicated series of oxidative enzymes. We know that the thyroid hormone increases the rate of biological oxidations, but detailed information is lacking as to how this effect is mediated by the enzymes. There are, of course, many other examples of the endocrine control of metabolic phenomena—the effects of insulin, of the adrenal cortical hormones and of the metabolic hormones of the pituitary gland on blood and tissue chemistry—but in all cases we are met with the same embarrassing lack of information concerning the mechanisms through which the hormones exert their effects.

A number of recent investigations have revealed a relationship between the endocrine status and the activity of selected enzymes. Atkinson and Elftman (1947) studied the mobilization by estrogen of alkaline phosphatase in the uterus; Pazos and Huggins (1945) demonstrated endocrine effects on the acid phosphatase content of dogs' prostates; Biddulph, Meyer and McShan (1946) found changes in

the succinic dehydrogenase system of the ovary which were related to the stage of the reproductive cycle; Kochakian (1946) correlated arginase activity with androgen stimulation; and many other examples could be cited. Moreover, evidence that the relationship between hormones and enzymes has causal significance has come from the demonstration that hexokinase may be inhibited and reactivated by different hormones (Price, Cori and Colowick, 1945). It now seems probable that intensive study of the endocrine regulation of enzyme systems will prove rewarding and will at least provide a beginning attack upon the mechanism of action of hormones.

The present experiments add several new instances in which enzymes have been shown to be hormonally controlled. In most of the examples described in the preceding section of this article, the new information is in good accord with prevailing concepts. The zona fasciculata of the adrenal cortex, the testis and thyroid gland are regulated by the hypophysis; it is not surprising therefore that phosphatase disappears after hypophysectomy and returns to these organs after replacement therapy. The uterus and seminal vesicles also afford information in good accord with the known regulation of these organs—phosphatase disappears alike after hypophysectomy or gonadectomy and returns upon injection of the appropriate hormones. On the other hand, the persistence of phosphatase in the parathyroid, pancreas and many non-endocrine organs is not surprising since the evidence for regulation of these organs by the hypophysis is at best equivocal.

Surprising facts come to light when other organs are considered. After hypophysectomy, phosphatase disappears from the zona fasciculata of the adrenal cortex but persists in the zona glomerulosa. Until recently the glomerulosa was regarded merely as the germinal zone of the cortex. Deane and Greep (1946) have shown, however, that the structure and apparent activity of this zone are maintained in the rat following hypophysectomy. Our observation that phosphatase also persists is in better accord with their findings than with the older hypothesis. Similarly, the maintenance of phosphatase in the corpus luteum is surprising. Insofar as is known, the endocrine activity of the ovary is completely dependent upon the hypophysis. A curious phenomenon occurs in the rat, however, in that the corpus luteum persists for long times after hypophysectomy (Greep, 1938). It would be interesting to examine the effect of procedures causing atrophy and disappearance of the corpus luteum upon its content of phosphatase.

The endocrine regulation of the mammary gland is not well understood. In normal and in ovariectomized rats, appropriate injection of ovarian hormones causes a rapid growth of this organ. After hypophysectomy, however, estrogen and progesterone exert but little effect on the mammary gland. Both of these operative procedures

depress the activity of phosphatase but do not abolish it. Collectively these facts suggest that some factor, as yet unknown, may participate in regulating the mammary gland.

We wish to mention also the correlation between the hypophysis and the phosphatase activity of the capillaries in muscle. It is, of course, well known that the carbohydrate metabolism of muscle is influenced by the pitutitary and adrenal cortical hormones, and that nitrogen retention and muscular hypertrophy are caused by androgens. Nevertheless, these effects are not striking morphologically, and one does not often think of striated muscle as an endocrine target organ. The present observations give visual indication of an endocrine regulation of muscle, and provide an end-point for further experiments designed to illuminate this most interesting problem.

SUMMARY

Alkaline phosphatase declines or disappears in the thyroid, testis and fasciculata of the adrenal cortex after hypophysectomy of the rat. After replacement therapy with whole pituitary powder, the phosphatase activity of these organs is restored to normal. The parathyroid gland, islets of Langerhans and many non-endocrine organs retain alkaline phosphatase in unchanged distribution and only slightly decreased concentration after hypophysectomy. Phosphatase also remains in the corpus luteum and zona glomerulosa of the adrenal gland after hypophysectomy.

Either hypophysectomy or gonadectomy causes phosphatase to disappear from the seminal vesicle and uterus. The normal appearance may be restored by injection of sex hormones in gonadectomized and pituitary powder in hypophysectomized rats. In the mammary gland, phosphatase declines in activity after both operative procedures, but a notable activity persists. The capillary endothelium of striated muscle also depends upon the integrity of the anterior pituitary gland.

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ASSOCIATION NOTICE

ANNOUNCEMENT OF THE 1949 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirty-First Annual Meeting of The Association for the Study of Internal Secretions will be held in the Chalfonte-Haddon Hall, Friday and Saturday, June 3, and 4, 1949, in Atlantic City, New Jersey.

We are informed by the hotel management that reservations will be difficult to secure on short notice; therefore, members are urged to make reservations at once with Chalfonte-Haddon Hall, giving time of arrival and length of stay in Atlantic City.

The scientific sessions will be held in the Viking Room, as formerly, and registration will be on the same floor. The annual dinner will be held in the Rutland Room, Friday, June 3rd. at 7 P.M., preceded by cocktails in the same room.

Those wishing to present papers, which will be limited to ten minutes, should send title and four copies of an abstract of not more than 200 words, to Doctor J. S. L. Browne, Royal Victoria Hospital, Montreal 2, Canada, not later than March 1, 1949. It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program.

Nominations for the Squibb and Ciba Awards and the Ayerst, McKenna and Harrison Fellowship should be made on special application forms, which may be obtained from the Secretary-Treasurer, Doctor Henry H. Turner, 1200 North Walker, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than March 15, 1949.

POSTGRADUATE COURSE IN ENDOCRINOLOGY

A postgraduate course in Endocrinology, sponsored by the Association for the Study of Internal Secretions, will be held at the Skirvin Hotel in Oklahoma City, February 21–26, 1949.

The faculty will consist of outstanding clinical and research endocrinologists of the United States and Canada. The program will consist of clinics and demonstrations and will be a practical one of equal interest to those in general medicine and the specialists.

The fee will be $100 for the entire course and applications will be accepted in the order received. Applications should be directed to Henry H. Turner, M. D., Secretary-Treasurer, 1200 North Walker, Oklahoma City, Oklahoma.

NOTES ON THE THIRTIETH ANNUAL MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirtieth Annual Meeting was held in the Palmer House, Chicago, Illinois, June 18 and 19, 1948.

Forty-seven papers were presented and forty-four papers were read by title. Total registration was 458, equally divided between members and non-members. Two hundred, twenty-two were present at the dinner at which the presidential address was given by Doctor C. N. H. Long.

Actions of general interest taken by the Council are as follows:

(1) It was the decision of the Council to hold a Postgraduate Assembly in Oklahoma City, February 21–26, 1949, similar to the one which was so well received in Los Angeles in 1948.

(2) The Council approved the indexing and publication of ENDOCRINOLOGY, Volumes 1 to 40, inclusive. These will be published by Mr. Charles C Thomas, in two editions, one including the indices of Volumes 1 to 25, and the other, Volumes 26 to 40.

(3) It was the Council's decision to publish the transactions of the American Goitre Association.

(4) The Council accepted with much regret the resignation of Doctor Earl T. Engle, Chairman of the Publications Committee, and appointed Doctor Warren O. Nelson to this office. Appreciation was expressed to Doctor Engle for his work as chairman of this committee.

(5) The Committee on Registry of Endocrine Pathology was re-appointed to negotiate with the Scientific Director of the American Registry of Pathology regarding the formation of an Endocrine Registry at the Army Institute of Pathology. A sum not to exceed $750.00 was appropriated for necessary expense for one year.

(6) It was voted to appropriate $250.00 for 1949 to the National Society for Medical Research.

(7) Due to increasing cost of labor, paper and all printing materials, and the enlargement of the Journals, the dues were increased to $11.00 per year which includes subscription to either of the Journals, with a combination offer of $16.00 for both Journals.

(8) The Council voted that the thirty-first Annual Meeting be held June 3 and 4, 1949, in Atlantic City, New Jersey.

(9) A list of the 1948–49 Officers, Council and Committees follows:

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President-Elect	Edward A. Doisy
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ASSOCIATION AWARDS FOR 1949

THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb & Sons Award of $1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner; 1941—Dr. Philip E. Smith; 1942—Dr. Fred C. Koch; 1943—no award was given; 1944—Dr. E. A. Doisy; 1945—Dr. E. C. Kendall; 1946—Dr. Carl G. Hartman; 1947—Drs. Carl F. and Gerty T. Cori; 1948—Dr. Fuller Albright. A special committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russel; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl G. Heller. The work cited may be either in the field of preclinical or clinical endocrinology. The Award is for $1,200.00. If within 24 months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, the Award will be increased to $1,800.00.

THE AYERST, McKENNA & HARRISON FELLOWSHIP

The Ayerst, McKenna & Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin, and in 1948 to Dr. Ernest M. Brown, Jr. This Fellowship is designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. The Fellowship may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend for the Fellowship will vary in accordance with the qualifications of the appointee, but will not exceed $2,500.00 The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. The nominee must present evidence or scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals; submit a program of proposed study; indicate one or more institutions where the proposed program shall be carried out; submit statements of approval from the investigators with whom he proposes to conduct his research.

Each member has the privilege of making one nomination for each award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to endocrinology and by a bibliography of the nominee's most important publications, and reprints, if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 15, 1949.

NEW BOOK

GENERAL ENDOCRINOLOGY. By C. Donnell Turner. Published by the W. B. Saunders Co., Philadelphia, Pa., 1948. v+604 pp. Price $6.75.

The concept of internal secretion, classically attributed to Claude Bernard, has found application in many physiological situations and has been amplified to include a multitude of substances which collectively comprise the chemical regulatory system of living organisms. A part of this regulatory system concerns the ductless glands, the study of which is the subject material of Endocrinology. Dr. Turner's book presents the basic and fundamental information of this latter field.

The point of view of the biologist rather than that of the physician has been emphasized throughout. The effort is to understand how systems work rather than how symptoms develop. Clinical information is not unduly slighted, but is placed in perspective with experimental observations and used insofar as it is of value in elucidating the way the endocrine system operates.

In any such presentation, an author has a hard time drawing the arbitrary line between the proper hormones and the closely related chemical regulators. Dr. Turner is no exception: he has included chapters on the plant hormones and the gastrointestinal principles but has treated cavalierly subjects such as acetylcholine, glucose and amino acids. This comment is not meant as a criticism, but indicates the difficulty one has in drawing sharp lines in endocrinology.

The author has chosen to present the basic and established facts and does not wander too far into the territory of controversial issues. This is undoubtedly a wise choice, particularly since the book was designed as a text for college students in biology and not for the more advanced students in professional schools. Nevertheless, the final result is a book somewhat more elementary than that ideally designed for medical students and physicians. The elementary student undoubtedly needs facts with which to think, and the elementary textbook should contain the agreed-upon factual background of the subject. For the more sophisticated student, on the other hand, training in how to think is advisable and perhaps no better method exists than to provide examples of how masters have thought and are thinking their way through controversial issues. Admirable as Dr. Turner's book is, it needs to be supplemented by another more advanced account for the more mature student.

These reflections concern matters related to the pedagogy of endocrinology as a whole, rather than the present book under review. Dr. Turner has defined the scope of his effort accurately and has acquitted himself excellently. The book is attractively printed and bound, contains a useful index and many informative drawings for which it was written, and it will find usefulness in many other areas as well.

ERRATA NOTICE

In the paper by Bartlett and Gaebler, entitled "Anterior Pituitary Growth Preparations: The Relationship Between Nitrogen Storage and Other Criteria of Activity" which appeared in Endocrinology 43: 334 (November) 1948, the following error has been noted:

Line 8, in footnote, under Table 5: "72 I. U." should read: "7 $\pm$ 2 I. U."